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The innate response to fungal infection

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Declarations

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the researchers or their publications. This dissertation has not in whole or in part been previously presented for a higher degree.

Dr Suzanne E. Elcombe

I certify that Suzanne Elizabeth Elcombe has spent the equivalent of at least nine terms in research work in the MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee and that she has fulfilled the conditions of the Ordinance General No. 14 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Dr J.S.C. Arthur
University of Dundee

Abbreviations

ABIN	A20-Binding Inhibitor of NFκB
ATF1	Activated Transcription Factor 1
BCL10	B Cell Lymphoma 10
BCR	B-Cell Receptor
BMDM	Bone Marrow Derived Macrophage
BSA	Bovine Serum Albumin
CARD9	Caspase-Recruitmant Domain 9
cDNA	Complementary DNA
COX	Cyclo-oxgenase
CRD	Carbohydrate Recognition Domain
CREB	cAMP Response Element Binding Protein
Ct	Threshold Cycle
DAG	Diacylglycerol
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulpoxide
DNA	Deoxyribonucleic Acid
DSTT	Division of Signal Transduction Therapy
DUSP1	Dual Specificity Phosphatase 1
ECL	Enhanced Chemiluminescence Reagent
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycoltetraacetic Acid

ERK	Extracellular-signal Regulated Kinase
FBS	Foetal Bovine Serum
g	Gram
Gfi1	Growth Factor Independent 1
h	Hour
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
IFN	Interferon
Ig	Immunoglobulin
IKKβ	Inhibitor of Nuclear Factor κB Kinase
IKKε	IkappaB Kinase ε
IL	Interleukin
IL-1R	IL-1 Receptor
IP_3	Inositol Triphosphate
IRAK	IL-1 Receptor Associated Protein Kinase
IRF3	Interferon Response Factor 3
ITAM	Immunoreceptor Tyrosine Activation Motif
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
kDa	Kilo-Dalton
KI	Knock In
KO	Knockout
LPS	Lipopolysaccharide

m	Milli
M	Molar
μ	Micro
MAL	MyD88 Adaptor Like Protein
MALT1	Mucosa-Associated Lymphoid Tissue Lymphoma Translocation Protein 1
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MEK	MAP Kinase or ERK Kinase
MEKK	MEK Kinase
min	Minute
MINCLE	Macrophage-Inducible C-type Lectin
MNK	MAPK Interacting Serine/Threonine Kinase
mol	Mole
mRNA	Messenger RNA
MSK	Mitogen and Stress Activated Protein Kinase
MyD88	Myeloid Differentiation Factor 88
n	Nano
NFAT	Nuclear Factor of Activated T-Cells
NF-κB	Nuclear Factor κB
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen Associated Molecular Pattern

PBS	Phosphate Buffered saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-Kinase
PIP_2	Phosphatidylinositol Biphosphate
PLC-γ	Phospholipase C-γ
Poly(I:C)	Polyinosine-Polycytidylic Acid
PRR	Pattern Recognition Receptors
PMA	Phorbol 12,13 Myristate Acetate
pPCR	Quantitative PCR
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
rRNA	Ribosomal RNA
RT	Reverse Transcription
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SIK	Salt Inducible Kinase
SILAC	Stable Isotope Labelling with Amino Acids in Cell Culture
STAT	Signal Transducer and Activator of Transcription
SYK	Spleen Tyrosine Kinase
TAB	TAK1 Binding Protein
TAK	Transforming Growth Factor-Beta-Activated Kinase
TBS	Tris Buffered Saline

TBS-T	Tris Buffered Saline with Tween
TCR	T-Cell Receptor
TEMED	N,N,N,N'-Tetramethylethylenediamine
TIR	Toll-IL-1 Receptor Domain
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
Tpl2	Tumour Progression Locus-2
TRAF	TNF-Receptor-Associated Factor
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-Related Adaptor Protein Inducing Interferon
Tris	Tris(hydroxymethyl)aminoethane
V	Volts
v/v	Volume/Volume
WT	Wild Type
w/v	Weight/Volume

Summary

In the healthy individual fungal infections are relatively benign, however in the rapidly increasing population of immunosuppressed patients fungal infections have become an increasing cause of morbidity and mortality. In the response to fungal pathogens, the innate immune system recognises a series of specific PAMPs via the Dectin-1 and TLR2 receptors, ultimately resulting in pro and anti-inflammatory cytokine production.

Following Dectin-1 activation, I show that blocking SYK activity with SYK inhibitor II prevents MAPK and NFκB signalling, causing a reduction in both pro and anti-inflammatory cytokine production. However, the clinically used inhibitor R406 (fostamatinib), which has been described as a SYK inhibitor, does not block these signalling pathways downstream of Dectin-1 activation, but is able to abolish cytokine production. As R406 has these effects in response to not only Dectin-1 ligand stimulation, but also TLR2 and TLR4 stimulation, it is likely that these events are the result of an off target effect of R406, and not a result of SYK inhibition. In line with this, R406 was found to inhibit multiple kinases in an *in vitro* kinase screening panel.

To investigate signalling further downstream of Dectin-1, I attempted to elicit the kinase responsible for ERK1/2 activation. For most stimuli, Raf-1 activates MEK1/2 which activates ERK1/2, however in response to TLR signalling the kinase Tpl2 is required to activate MEK1/2. I show that the kinase responsible for ERK1/2 activation downstream

of Dectin-1 is not Tpl2, but is an unidentified off target effect of the Tpl2 small molecule inhibitor SHN681.

MSK1 and 2 have previously been shown to be important in limiting inflammatory cytokine production by macrophages in response to the TLR4 agonist LPS. This is in large part due to the ability of MSKs to regulate the production of the anti-inflammatory cytokine IL-10. In this thesis I show that MSKs are activated in macrophages by fungal ligands, including the Dectin-1 specific agonists curdlan and depleted zymosan, via the ERK1/2 and p38α MAPK pathways. Further, I show that although MSKs regulate Dectin-1 induced IL-10 transcription, this does not significantly affect pro-inflammatory cytokine production. This is in direct contrast to the inhibition of these pro-inflammatory cytokine that we see post LPS stimulation. Investigating further, I show that although IL-10 secreted in response to zymosan is unable to suppress pro-inflammatory cytokine production, it is still able to promote STAT3 phosphorylation. I suggest that Gfi1 is involved as I show that LPS can induce high levels of Gfi1 expression, whereas zymosan does not induce Gfi1. One possible explanation would be that without Gfi1 to inhibit PIAS3, PIAS3 is binding the activated STAT3 and not allowing it to bind to DNA. This would result in STAT3 being unable to function, and hence no repression of pro-inflammatory cytokine expression would occur, regardless of the level of IL-10 present.

Finally, I show that activation of Dectin-1 in a SYK dependent fashion resulted in macrophage switching to a regulatory macrophage phenotype. As regulatory macrophages are thought of as essentially anti-inflammatory, this may help explain why

many people suffer commensal fungal infections that can persist for long periods of time without developing the classical inflammatory signs of infection.

1. Introduction

1.1 The Immune System – An Overview

The body is protected from infectious agents and pathogens by its immune system. To be effective the immune system must have components that recognise a potential pathogen, effector cells that combat the pathogen and an immunological memory to ensure rapid recognition and elimination in future encounters. There must also be the ability for the immune system to self-regulate, to prevent auto-immunity.

The human immune system comprises of two main components – the innate immune system and the adaptive system. The cells of the innate immune system include macrophages, dendritic cells (DCs) and neutrophils, all of which are programmed to be able to detect invariant features of invading pathogens. Innate immune responses occur rapidly on exposure to pathogen. The adaptive immune system comprises T lymphocytes and B lymphocytes. These cells employ non-germ line encoded receptors which allows very specific pathogen recognition. However, it means that their response although much more efficient at pathogen elimination than the innate system can take days to generate. A subset of previously activated lymphocytes can last long after an initial infection has been combated, and hence are capable of generating immunological memory (Iwasaki and Medzhitov 2010).

In this thesis I will focus mainly on the response of the innate immune system to fungal pathogens.

1.2 Pattern Recognition Receptors

Recognition of a pathogen is the first step in a body's defence against infection. In the innate immune system, this is achieved through germ-line encoded pattern recognition receptors (PRRs) that recognise highly conserved microbial patterns known as Pathogen Associated Molecular Patterns (PAMPs).

PRRs can be divided into secreted, cytosolic and transmembrane receptors. Secreted PRRs bind to pathogens, activate the complement system and opsonise pathogens for phagocytosis by macrophages and neutrophils. Cytosolic PRRs, for example RIG-I-like helicases, detect viral pathogens by responding to foreign DNA and foreign RNA, and NOD like receptors that detect microbial pathogens and stress signals. Transmembrane PRRs, examples of which include Toll-like receptors (TLRs) and C-type lectins, are expressed on plasma membranes and recognise PAMPs that are available on the pathogens cell surface. Transmembrane receptors, notably TLRs, can also be expressed on endosomal, phagosomal and lysosomal membranes where they can detect the presence of many foreign ligands including DNA, RNA and components of pathogen cell walls (Iwasaki and Medzhitov 2010).

1.3 Fungal Infection

Fungal infections in healthy individuals are relatively benign, but in the immunocompromised they are a major source of morbidity and mortality (Warnock 2007). This at risk population includes patients undergoing treatment for malignancies or

autoimmune conditions, recipients of solid organ or haematopoetic stem cell transplants, people with human immunodeficiency virus (HIV), burns patients and individuals whom have long-term indwelling medical devices.

Over the last 30 years the number of cases of invasive fungal infections has risen dramatically. This is largely due to the decreasing mortality of those who are chronically immunosuppressed, notably with improved treatments for HIV, the increasing success of organ transplantation, and more intensive treatments for autoimmune diseases resulting in dramatic prolonged immunosuppression (Warnock 2007).

1.4 The Innate Response to Fungal Infection

In the innate response to fungal pathogens, there are a number of PRRs that are potentially involved. These include the Toll-like receptors (TLRs), the C-type lectin receptors, the mannose receptor and the complement receptor 3 (Brown 2006). β-glucan is a major constituent of fungal cell walls, and following the discovery that Dectin-1 was a major β-glucan receptor on macrophages (Brown and Gordon 2001), many strategies were undertaken to determine the relative significance of each PRR in the recognition of fungal pathogens and the effect on cellular signalling. This was initially through the use of zymosan, a reagent prepared from *Saccharomyces cerevisiae,* which predominately contains β-glucan and mannan (Di Carlo and Fiore 1958), as a model fungal stimulus. Later the Dectin-1 specific ligand depleted zymosan (Ikeda, Adachi et al. 2008) and the purified β-1,3glucan curdlan were also used.

Results following the use of a Dectin-1 neutralising antibody and Dectin-1 knock out mouse strengthened the evidence for the role of Dectin-1 in the recognition of the β-glucan in zymosan by primary macrophages (Brown, Taylor et al. 2002). TLR2 has also been shown to be heavily involved in the response to zymosan.

1.5 C-type Lectin Receptors

The C-type lectin receptor family comprises a large group of proteins that all contain a conserved carbohydrate recognition domain that provides a highly variable interface for binding carbohydrate structures (Drickamer 1989).

The C-type lectin receptor family is divided into 17 groups based on phylogeny and domain organisation, and although the C-type lectin family were described originally for their calcium dependent lectin activity, it has since been shown that many members of this family do not require calcium nor bind carbohydrate ligands (Zelensky and Gready 2005)

Despite all family members containing the highly conserved carbohydrate recognition domain, the C-type lectin receptors are all functionally diverse with roles in cell adhesion, complement activation, pathogen recognition and phagocytosis (reviewed in (Kerrigan and Brown 2010). Several C-type lectin receptors including Dectin-1, Dectin-2, MINCLE (macrophage-inducible C-type lectin) and the mannose receptor are involved in innate anti-fungal immunity.

1.5.1 Dectin-1

Dectin-1 is a C-type lectin type II transmembrane receptor that was first discovered as a dendritic cell-specific receptor that interacted with T cells (Ariizumi, Shen et al. 2000). Dectin-1 is highly expressed on neutrophils, inflammatory macrophages and alveolar macrophages, with lower expression on resident macrophages and DCs (Taylor, Brown et al. 2002). It has also been shown to be present on B-cells, although its exact role here is not clear (Willment, Marshall et al. 2005). Dectin-1 is the predominant macrophage receptor for β-glucan (Brown and Gordon 2001), and with β-glucan being essential for maintaining fungal cell structure integrity, it can comprise up to 50% of the fungal cell wall (Calderone and Braun 1991).

1.5.2 Dectin-1 Knock Out Mouse

Dectin-1 knockout mice are viable with no apparent phenotype exhibited when they are maintained in specific pathogen free conditions. Several groups have now reported the response of the Dectin-1 knockout mouse to *in vivo* fungal infections. When exposed to *Pneumocystis carinii* they showed an early impairment in lung clearance, but ultimately cleared the organism as well as wild type mice (Saijo, Fujikado et al. 2007). On exposure to *Cryptococcus neoformans* the Dectin-1 deficient mice did not differ in their response from wild type mice. However, on exposure to *Aspergillus fumigatus*, the deficient mice were unable to produce significant pro-inflammatory cytokines, and had delayed fungal clearance, lower neutrophil lung recruitment and ultimately severe lung dysfunction rapidly leading to death (Werner, Metz et al. 2009).

The outcome following infection with the common fungi *Candida albicans* is less clear, with the literature split in favour of Dectin-1 being required for protection against disseminated candidiasis (Taylor, Tsoni et al. 2007), and there being no difference in susceptibility to C*andida albicans* infection between the Dectin-1 deficient and wild type mice (Saijo, Fujikado et al. 2007). A likely explanation for this difference is that the two groups used different strains of *C.albicans* to inoculate the mice (Drummond and Brown 2011). Another likely conflicting factor is the fact that both groups have made their own Dectin-1 knockout mice on different mouse backgrounds. Thus, so far mouse studies have not been able to give a clear role for Dectin-1 in candidiasis.

1.5.3 Mutations in Human Dectin-1

In 2009, the first functional Dectin-1 polymorphism was reported in a Dutch family whom suffered with chronic or recurring onychomycosis or recurrent vulvovaginal candidiasis (Ferwerda, Ferwerda et al. 2009). The authors found that 3 sisters all have a homozygous single nucleotide polymorphism within Dectin-1 (rs16910526, exon 6, chromosome 12). The mutation is an A to C base change within the carbohydrate recognition domain (CRD) of Dectin-1, and results in a change of amino acid 238 from tyrosine to a stop codon (Tyr238X). This mutation truncates the CRD by 9 amino acids. Macrophages taken from individuals with this mutation expressed very low levels of the Dectin-1 receptor on the cells surface, and that the macrophages were almost unresponsive to β-glucans, producing a very impaired cytokine response. However, these patients' neutrophils were still able to phagocytose and kill *C.albicans* as normal, which

explains why these patients only suffer from onychomycosis and vulvovaginal candidiasis, and do not develop invasive fungal infections (Ferwerda, Ferwerda et al. 2009).

Subsequently, a cohort of patients with haematological malignancies was studied (Plantinga, van der Velden et al. 2009), and it was found that patients who were heterozygous for the Dectin-1 Tyr238X mutation were more likely to be colonised with *C.albicans* (oral and gastrointestinal) than patients without this polymorphism. Surprisingly, the presence of the Dectin-1 polymorphism did not result in higher incidence of systemic candidiasis, but this maybe explained by the routine use of fluconazole to eradicate colonisation in these high risk patients. Additionally, there were no patients who were homozygous for the Dectin-1 mutation in this cohort. However, macrophages from the heterozygous patients showed a significant decrease in Dectin-1 receptor expression on the cell surface compared to the wild type macrophages (Plantinga, van der Velden et al. 2009); hence heterozygosity for the mutation does have functional significance. This study again shows that adequate Dectin-1 receptors are necessary to prevent colonisation of mucosal surfaces by *Candida*, but are not necessary in the prevention of disseminated invasive candidiasis.

1.5.4 Other C-type Lectin Receptors

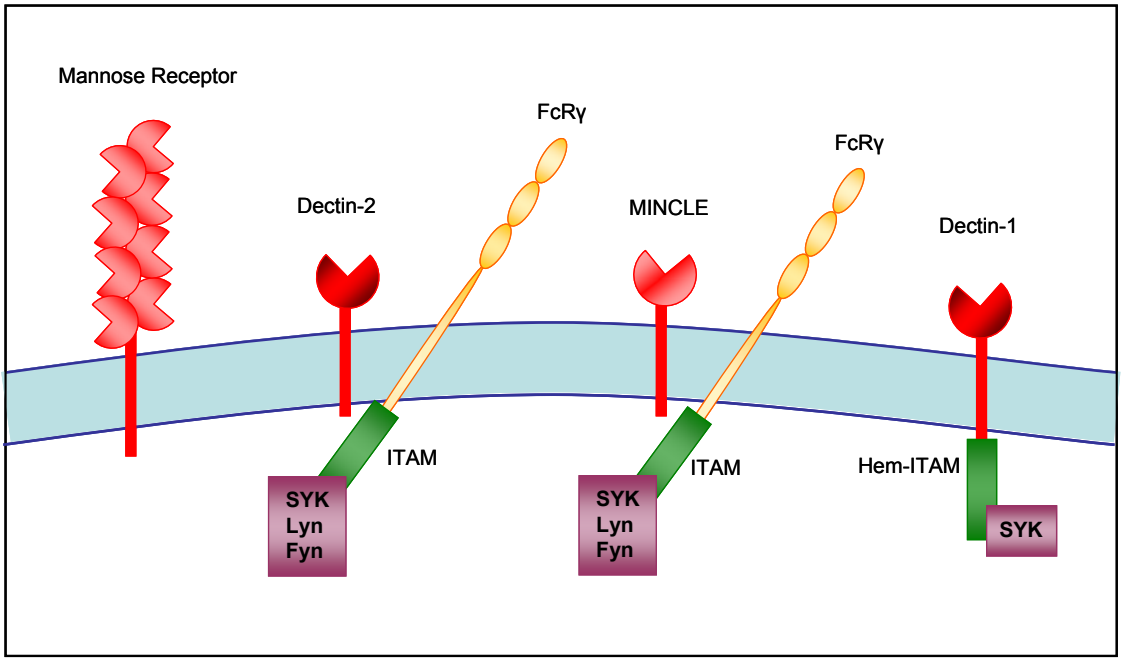
The other main C-type lectin receptors involved in the innate immune response to fungi are Dectin-2, MINCLE (macrophage-inducible C-type lectin) and the mannose receptor (illustrated in figure 1).

Dectin-2 is a type II transmembrane receptor that is predominately expressed on tissue macrophages and inflammatory monocytes, recognising high mannose structures contained in many fungal pathogens (McGreal, Rosas et al. 2006). Dectin-2 has a very short cytoplasmic domain, lacking any obvious signalling motif, and so once activated must associate with the FcRγ adaptor, which contains an ITAM, to then enable signalling, which occurs via the SYK-CARD9 pathway (Sato, Yang et al. 2006; Bi, Gojestani et al. 2010). Dectin-2 knock out mice showed virtually no cytokine production in response to fungal α-mannan, and the yeast forms of *C.albicans* induced IL-1β and IL-23 secretion in a Dectin-2 dependent manner (Saijo, Ikeda et al. 2010). This suggests that Dectin-2 is important in detecting and responding to the less immuno-vigorous yeast forms of fungus that Dectin-1 is less able to detect.

MINCLE is a type II transmembrane protein that is upregulated once a macrophage becomes activated, primarily recognising fungal α-mannan and mycobacterial cord factor (Matsumoto, Tanaka et al. 1999). Like Dectin-2, MINCLE has a very short cytoplasmic tail and so must also associate with the FcRγ adaptor in order to signal through the SYK-CARD9 pathway (Yamasaki, Ishikawa et al. 2008). Primary macrophages from MINCLE knockout mice showed reduced production of pro-inflammatory cytokines, notably TNF-α, in response to *C.albicans* (Wells, Salvage-Jones et al. 2008). In *vivo*, the MINCLE knock out mice show a significantly increased susceptibility to systemic candidiasis (Wells, Salvage-Jones et al. 2008).

The mannose receptor is a type I transmembrane protein that is expressed predominately by macrophages, both within the endocytic pathway and in a released soluble form as a cytosolic receptor. The mannose receptor has a wide variety of ligands such as terminal mannose, sulphated sugars, frucose, glucosamine and a number of endogenous proteins, and as such can induce a range of cellular responses depending on the exact stimuli. To date, the mechanisms of intracellular signalling downstream from the mannose receptor have not been elucidated. Mannose receptor knockout mice show no increase in susceptibility to infection by *C.albicans* (Lee, Zheng et al. 2003).

Figure 1 Illustration of the C-type Lectin Receptors Involved in the Innate Response to Fungi.
Adapted from (Dostert and Tschopp 2007)



1.5.5 CARD9 Knockout Mouse

The caspase-recruitment domain protein 9 (CARD9) has been shown to be essential for the propagation of signalling downstream of the C-type lectin receptors, as detailed later in section 1.8. CARD9 knockout mice have been generated which are viable with no apparent phenotype exhibited when they are maintained in specific pathogen free conditions (Gross, Gewies et al. 2006). The CARD9 deficient mice were shown not to have any defect within T- and B-cell differentiation and activation for adaptive immunity. In response to the bacterial pathogen *Staphylococcus aureus* the CARD9 knockout mice cleared the infection as effectively as the wild type mice. However, the CARD9 knockout mice showed significantly increased susceptibility to infection by *Candida albicans*, with significantly increased mortality rates and massively increased fungal burdens in the kidneys, liver and lungs when compared to wild type mice (Gross, Gewies et al. 2006).

1.5.6 Mutations in Human CARD9

DNA analysis from 36 members of a large consanguineous Iranian family identified a homozygous mutation in exon 6 of CARD9 that results in a loss of function mutation due to a premature stop codon (Q295X) in the coding sequence. All of the homozygous individuals had recurrent oropharyngeal and/or vulvovaginal candidiasis, and 2 members of the family had previously developed fatal meningeal candidiasis (Glocker, Hennigs et al. 2009). This suggests that the CARD9 mutation confers a stronger phenotype that the Dectin-1 mutation, which could be in part due to the fact that CARD9 also relays signals from other C-type lectin receptors. However, when comparing these studies we must bear

in mind the consanginuity of the Iranian family and as such other genetic defects may have contributed to the more severe phenotype seen.

1.6 Toll-Like Receptors

Toll-like receptors (TLRs) are probably the most studied of all the PRRs. The receptor protein Toll was first identified in *Drosophila meglanogaster* as the gene that was responsible for controlling dorso-ventral patterning in the embryo (Anderson, Bokla et al. 1985). It was noted that activation of the Toll receptor by its ligand (Spatzle) activated a signalling cascade which ultimately activated Dorsal, the *Drosophila* version of the mammalian NFκB (reviewed in (Qureshi and Medzhitov 2003). In 1996 it was discovered that mutations in *Drosophila* Toll resulted in the inability of adult flies to defend against fungal pathogens (Lemaitre, Nicolas et al. 1996). Subsequently, homologs of Toll, called Toll-like receptors, have been found in mammals. The first indication that TLRs were important in mammalian immunity came from studies looking at two LPS resistant strains of mice, C3H/HeJ and C57/10ScCr (Poltorak, He et al. 1998). Poltorak *et al* found that the mouse strain C3H/HeJ had a missense mutation in the third exon of the TLR4 gene, resulting in the replacement of proline by histidine at position 712 of the polypeptide chain, and that C57/10ScCr mice were homozygous for a null mutation of TLR4, hence providing the first evidence that a functional TLR4 is required to recognise LPS (Poltorak, He et al. 1998).

To date there have been 13 TLRs described, 10 of which (TLR1-10) are expressed in humans, and 12 of which (TLR1-9 and TLR11-13) are expressed in mice (Kawai and

Akira 2010). All TLRs are type 1 transmembrane glycoprotein receptors, and each TLR recognises a distinct set of evolutionarily conserved PAMPs that are not found in healthy vertebrate cells (Akira, Uematsu et al. 2006), these are summarised in table 1.

The first TLR knockout mouse described was the TLR4 knockout mouse. The authors showed that macrophages deficient in TLR4 were unable to activate NFκB and failed to produce any detectable TNFα in response to LPS stimulation (Hoshino, Takeuchi et al. 1999). Following this, the TLR2 knockout mouse was produced. The TLR2 deficient mice responded to LPS to the same level as wild type mice, but were hyporesponsive to peptidoglycan, a major constituent of Gram positive bacteria cell wall (Takeuchi, Hoshino et al. 1999). TLR2 knockout mice are highly susceptible to infection by the Gram positive bacteria *Staphlococcus aureus*, being able to produce significantly less cytokines than the wild type (Takeuchi, Hoshino et al. 2000).

Regarding the TLR knockout mouse response to fungal pathogens, it is reported that neither the TLR2 nor the TLR4 knockout mice were more susceptible to infection by *Aspergillus fumigatus* than the wild type mice (Dubourdeau, Athman et al. 2006). Further, TLR2 and TLR4 knockout mice both respond as wild type mice following infection with *Cryptococcus neoformans* (Nakamura, Miyagi et al. 2006). In response to *Candida albicans* there was no difference in susceptibility to primary candidiasis in the TLR2 knockout mice when compared to wild type, however interestingly the TLR4 knockout mice did show increased susceptibility to infection (Bellocchio, Montagnoli et al. 2004). However, in conflict to this, it has also been reported that the TLR2 knockout

mice are also more susceptible to *Candida albicans* than wild type mice (Netea, van der Graaf et al. 2002).

The individual TLR knockout mice results are confounding. It is possible that when one TLR has been knocked out another may compensate, and so another way to investigate the role of TLRs in fungal infection is to look at the MyD88 knockout mice. TLR signalling is discussed later in section 1.8.3, however MyD88 is an essential component for signalling downstream of all TLRs, with the exceptions of TLR3 and TLR4, and without it there can be no compensation from other MyD88 signalling TLRs. The MyD88 knockout mice consistently show that they are more susceptible than the wild type to infections by *Candida* (Bellocchio, Montagnoli et al. 2004) (Marr, Balajee et al. 2003). The effect of the MyD88 knockout in response to *Aspergillus fumigatus* differs greatly with reports varying from showing no difference in susceptibility to infection in the knockouts (Marr, Balajee et al. 2003; Bellocchio, Montagnoli et al. 2004), to delayed clearance of *Aspergillus* (Bretz, Gersuk et al. 2008), through to failure to produce any pro-inflammatory response in the MyD88 knockout mice (Mambula, Sau et al. 2002). These reports are conflicting and many factors are likely to be contributing, such as differences in the sources of the animals, the exact preparation of fungus used and the time scales of studies.

In humans, screening of groups with candidiasis has shown that TLR2 mutation Arg753Gln is a risk factor for *Candida* sepsis (Woehrle, Du et al. 2008), and that TLR1 polymorphisms resulting in impaired function of TLR1 are associated with decreased

cytokine responses and an increased susceptibility to candidemia (Plantinga, Johnson et al. 2012). All studies however are very small and struggle to reach statistical significance.

Table 1 TLR Receptors and Their Ligands

Toll-Like Receptor	Specific Ligands
TLR1:TLR2 heterodimer	Bacterial lipoproteins (Triacylated lipoproteins from Gram negative bacteria)
TLR2	Peptidoglycan and Lipoteichoic acids (Gram positive bacteria) Lipomannans (fungi and mycobacteria)
TLR2:TLR6 heterodimer	Bacterial lipoproteins (Diacylated lipopeptides from Gram positive bacteria)
TLR3	Double-stranded RNA (viruses)
TLR4	Lipopolysaccharide (LPS) (a cell wall component of Gram negative bacteria)
TLR5	Flagellin (element of bacterial flagella)
TLR7	Single-stranded RNA (viruses)
TLR8	Single-stranded RNA (viruses)
TLR9	DNA with non methylated CpG (bacteria and herpes viruses)
TLR10	Unknown
TLR11 (mouse only)	Profilin and Profilin-like proteins (uropathogenic bacteria)
TLR12 (mouse only)	Unknown
TLR13 (mouse only)	Unknown

1.7 Dectin-1 and TLR Synergy

Dectin-1 and TLR2 can act synergistically, and produce a larger cytokine and pro-inflammatory response than that achieved by either receptor individually (Gantner, Simmons et al. 2003; Ferwerda, Meyer-Wentrup et al. 2008). It is suggested that Dectin-1 enhances the TLR2 signal via a CARD9 dependent mechanism, as it is shown that CARD9 deficient bone marrow derived macrophages (BMDM) have a defect in TNFα production following zymosan stimulation (Goodridge, Takahiro et al. 2009).

The relative contribution of Dectin-1 versus TLR2 in the *in vivo* response to *Aspergillus fumigatus* has been extensively studied. As detailed earlier, the Dectin-1 knockout mouse in response to *A. fumigatus* was unable to produce significant pro-inflammatory cytokines, and had delayed fungal clearance (Werner, Metz et al. 2009). The role of Dectin-1 is supported by a further study using a soluble Dectin-1 fusion protein to block Dectin-1 in alveolar macrophages. By blocking Dectin-1 the authors saw a reduction in pro-inflammatory cytokine levels and an increase in fungal burden (Steele, Rapaka et al. 2005). In TLR2 knockout mice, the authors saw a minimal reduction in pro-inflammatory cytokines in response to *A. fumigatus,* with only a slight reduction in TNFα production showing statistical significance (Steele, Rapaka et al. 2005). This enhancing, but not essential, role of TLR2 in the macrophage response to *A. fumigatus* is supported by the data from the MyD88 knockout mice. TLR2 requires MyD88 to signal following activation, and hence MyD88 knockout is another useful way to remove the TLR2 contribution. The MyD88 knockout removes all input from the other MyD88 signalling TLRs (that is all but TLR3 and TLR4), and so removes the possibility of any

compensation from other TLRs that might be seen in the TLR2 only knockout. It is reported that the MyD88 knockout mouse, in response to *A. fumigatus*, shows increased fungal burden, delayed lung clearance and delayed resolution of inflammation (Bretz, Gersuk et al. 2008). It is therefore apparent that for optimum macrophage inflammatory response to *A. fumigatus* you require Dectin-1, but the potentially redundant pathway of TLR2 is important for mediating the inflammatory response.

Dectin-1 is reportedly the sole receptor on macrophages for β-glucans (Saijo, Fujikado et al. 2007); however, these glucans are not readily exposed on fungal hyphae but are seen predominantly at bud scars and on conidia (Gantner, Simmons et al. 2005). It has been suggested that through differential PRR recognition macrophages can tailor their immune response to the invasiveness of pathogen encountered. For example, in the response to *Aspergillus fumigatus,* macrophages bind and ingest the resting conidia effectively without activating Dectin-1 and with little inflammatory response (Gersuk, Underhill et al. 2006). However, mature conidia and germ tubes do show a large amount β-glucan on their surface and as a result activate Dectin-1, resulting in large secretions of proinflammatory cytokines (Gersuk, Underhill et al. 2006). TLR2 also recognises the hyphal form of *Aspergillus fumigatus*, and whilst Dectin-1 binding is shown to only augment the TLR2 mediated IL-6 and IL-12 secretion in response to *Aspergillus fumigatus*, it is absolutely required for TNFα production (Gersuk, Underhill et al. 2006).

1.8 Signalling Downstream of Immune Receptors

1.8.1 General Principles of Signalling

The enzymes most commonly associated with receptor activation and intracellular signalling pathways are the protein kinases. Protein kinases are a large group of enzymes that catalyse the covalent attachment of a phosphate group to a protein. This is a reversible process, known as protein phosphorylation. Protein kinases can be divided into two main groups, tyrosine kinases which phosphorylate tyrosine residues, and serine/threonine kinases that phosphorylate either serine or threonine residues. An exception to this are the STE7 family, which can phosphorylate both threonine and tyrosine residues (Manning, Whyte et al. 2002).

To allow this signal to be switched off, a group of enzymes called protein phosphatases are able to remove the phosphate group from phosphotyrosine or from phosphoserine/phosphotyrosine. This specific phosphorylation and dephosphorylation is critical to the regulation of signalling pathways and is a process that is used in many cellular systems as a way of controlling the activity of enzymes, transcription factors and many other proteins.

1.8.2 ITAM Linked Signalling Pathway

Many receptors of the immune system, notably the B-cell receptor (BCR), the T-cell Receptor (TCR), the Fc Receptor and some of the C-type lectin receptors, contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tail. An

ITAM consists of a short amino acid sequence containing a duplicate of the sequence YxxL/I with 6 to 12 intervening residues. Each ITAM contains two tyrosine residues that become phosphorylated when the receptor binds to its ligand; this in turn allows the recruitment of signalling molecules that contain tandem SRC homology 2 domains (Underhill and Goodridge 2007).

In macrophages, Dectin-1 is one of the receptors that signal through an ITAM-like motif (hemi-ITAM). Dectin-1 has an ITAM-like motif because the two tyrosines contained in its cytoplasmic tail are positioned in a similar fashion to those in an ITAM but the N-terminal tyrosine resides in a YxxxL motif (YxxxI in mice), opposed to the ITAM sequence of YxxL (Kerrigan and Brown 2011). Ligand binding to the Dectin-1 receptor leads to phosphorylation of the ITAM-like domain which then associates with spleen tyrosine kinase (SYK) (Rogers, Slack et al. 2005).

1.8.2.1 Spleen Tyrosine Kinase

SYK is a 72kDa tandem SRC homology 2 domain-containing tyrosine kinase. SYK is best known for its role in signalling downstream of the classical immune receptors, notably the BCR, TCR and the Fc receptors (Mocsai, Ruland et al. 2010). However, SYK has been shown to mediate signalling by other receptors, including integrins (Mócsai, Zhou et al. 2002) and C-type lectins (Kerrigan and Brown 2010), and has been implicated in many non-immune roles, for example bone metabolism, platelet function and tumour suppression (reviewed in (Mocsai, Ruland et al. 2010).

Despite its far reaching biological roles, SYK has perhaps been most studied downstream of the BCR. Stimulation of the BCR causes cross-linking of the receptors, activating the membrane bound src-family tyrosine kinases, Fyn, Blk and Lyn. These kinases then in turn phosphorylate the ITAMs on the cytoplasmic tails of Igα and Igβ, which recruit and activate SYK. As there are usually many receptor complexes clustered together, many SYK molecules can be bound in close proximity, and are therefore able to further phosphorylate and activate each other to promote enhanced signalling. SYK can activate phospholipase C-γ (PLC-γ), which then cleaves phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG then activates protein kinase C, small G proteins activate the MAPK cascade, and IP_3 increases intracellular calcium activating calcineurin, which in turn activates nuclear factor of activated T-cells (NFAT). All of which ultimately controls the induction of specific cytokine gene transcription (reviewed in (Mocsai, Ruland et al. 2010).

1.8.2.2 Dectin-1 Signalling Downstream of SYK

As discussed, Dectin-1 phosphorylation upon ligand binding leads to a direct interaction between Dectin-1 hem-ITAM and SYK (Rogers, Slack et al. 2005). This is thought to initiate a signalling cascade that induces a scaffold comprising of the caspase-recruitment domain protein CARD9 with the adaptor proteins BCL10 and MALT1 (Goodridge, Takahiro et al. 2009). It has been shown that the CARD9-BCL10-MALT1 scaffold is required to activate the IkappaB kinase (IKK) complex, which ultimately leads to

activation of transcription factors including NFκB, and ultimately cytokine production (Gross, Gewies et al. 2006).

A second, unclear, signalling pathway occurs from SYK following Dectin-1 ligand binding– whereby SYK triggers reactive oxygen species generation, which in turn causes NLRP3 inflammasome activation (Gross, Poeck et al. 2009). This is necessary for the activation of caspase-1, which is then able to cleave pro-IL-1β to IL-1β allowing the cytokine to become active and secreted.

A further signalling pathway has also been described whereby Dectin-1, independently of SYK, activated the serine-threonine kinase Raf-1. The researchers added a neutralising antibody to Dectin-1 or piceatannol to inhibit SYK. They showed that by adding antibody to Dectin-1 they could block Raf-1 phosphorylation, but Raf-1 was still able to be phosphorylated in the presence of piceatannol (Gringhuis, Dunnen et al. 2009). This implies that Dectin-1 is capable of activating Raf-1 independently of SYK. This pathway was supported by data using RNA-mediated interference to suppress Raf-1, which showed that without Raf-1 the expression of IL-10, IL-12 (p40), IL-6 and IL-1β was decreased (Gringhuis, Dunnen et al. 2009).

The role of MAP kinase signalling downstream of Dectin-1 is not yet fully understood. It has been shown that ERK1/2 activation in response to zymosan is independent of the TLR2/MyD88 pathway (Slack, Robinson et al. 2007) and that ERK1/2 inhibition reduces the amount of IL-10 produced in response to zymosan (Dillon, Agrawal et al. 2006). It

has not been demonstrated as yet whether ERK1/2 is activated, as TLRs are, by Tpl2, or by Raf-1 downstream of Dectin-1.

1.8.2.3 Genetic Loss or Inhibition of SYK

The systemic loss of SYK is perinatally lethal due to defects in endothelial cells within the vasculature, and so therefore it is not possible to study adult total SYK deficient mice. Instead, the effect of SYK deficiency in haematopoeic cells has been primarily studied following the reconstitution of irradiated mice with SYK knockout foetal liver cells. This has shown that the absence of SYK causes failure of B-cell maturation and the total loss of immune receptor signalling (Cheng, Rowley et al. 1995; Turner, Mee et al. 1995). Conditional SYK knockout mice have been made, using Cre recombinase to conditionally delete SYK in the myeloid cells only. In these mice, it has been shown that *in vitro* SYK deficient neutrophils are unable to secrete cytokines and generate reactive oxygen species, and *in vivo* the SYK bone marrow chimera mice have significant impairment in their ability to successfully kill *Staphlococcus aureus* (Van Ziffle and Lowell 2009). However, the conditional mice seem to be protected from the development of autoimmune conditions, as they are shown to be more resistant to the development of autoantibody induced experimental arthritis, with the ability to block both macroscopic and microscopic signs of arthritis (Jakus, Simon et al. 2010).

In humans, rheumatoid arthritis (RA) has been associated with autoantibodies directed against antigens such as type II collagen and heat shock proteins in addition to the ubiquitous rheumatoid factor, which recognises the Fc fragment of IgG (Steiner and

Smolen 2002). It is therefore not surprising that these immune complexes lead to inflammatory cytokine release within joints, causing further synovitis and ultimately further joint destruction. As these autoantibody immune complexes are abnormal, they stimulate the Fc receptors on various haematopoietic cells. The activated Fc receptors contain an ITAM motif which when phosphorylated activates SYK, resulting in cytokine gene expression and an inflammatory response (Braselmann, Taylor et al. 2006). SYK inhibitors are therefore a potential therapeutic target for preventing inflammation in rheumatoid arthritis. The only current clinical SYK inhibitor R788, also known as fostamatinib disodium, (prodrug of the active metabolite R406), has been shown to reduce the severity of autoimmune arthritis in mouse models (Braselmann, Taylor et al. 2006; Pine, Chang et al. 2007). Following this, a 12 week randomised placebo-controlled pilot human study was undertaken in people with active RA despite methotrexate therapy. A significant benefit was seen by the first week in those patients taking R788, with significant decreases in IL-6 and a marked improvement in symptoms (Weinblatt, Kavanaugh et al. 2008). A second randomised placebo controlled trial was undertaken in a cohort of patients that had refractory RA, that is had failed all treatments including biological agents such as anti-TNF therapy, anakinra, abatacept and/or rituximab. In this refractory cohort there was no difference between the R788 treatment and placebo groups (Genovese, Kavanaugh et al. 2011). This is not entirely surprising as it is likely that such severe and unresponsive arthritis has many multi-factorial inflammatory driving processes. Large phase III trials with R788 in RA are currently underway.

To date there have been 5 phase II clinical studies using R788, and although RA represents the most numerous and notable clinical studies (totally 3 of the 5), it has also been trialled in other autoimmune diseases, with phase II trial success in the treatments of autoimmune thrombocytopenia and lymphoma (McAdoo and Tam 2011).

1.8.3 TLR Activation and Signalling

The TLRs can be divided into three groups according to the adaptor proteins they associate with upon activation. There are 4 different adaptor proteins used by mammalian TLRs: Myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL), TIR domain containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). TLRs 5, 7, 8 and 9 all interact with the adaptor protein MyD88 alone. TLR2 requires MyD88 and MAL. TLR3 interacts only with TRIF, whilst TLR4 is in the unique position of interacting with both MyD88/MAL and TRIF/TRAM complexes (reviewed in (Kawai and Akira 2010). Interactions of TLRs with TRIF leads to the generation of both a type I interferon response and inflammatory cytokines, whereas MyD88 signalling alone induces primarily inflammatory cytokines (McCoy, Macdonald et al. 2007).

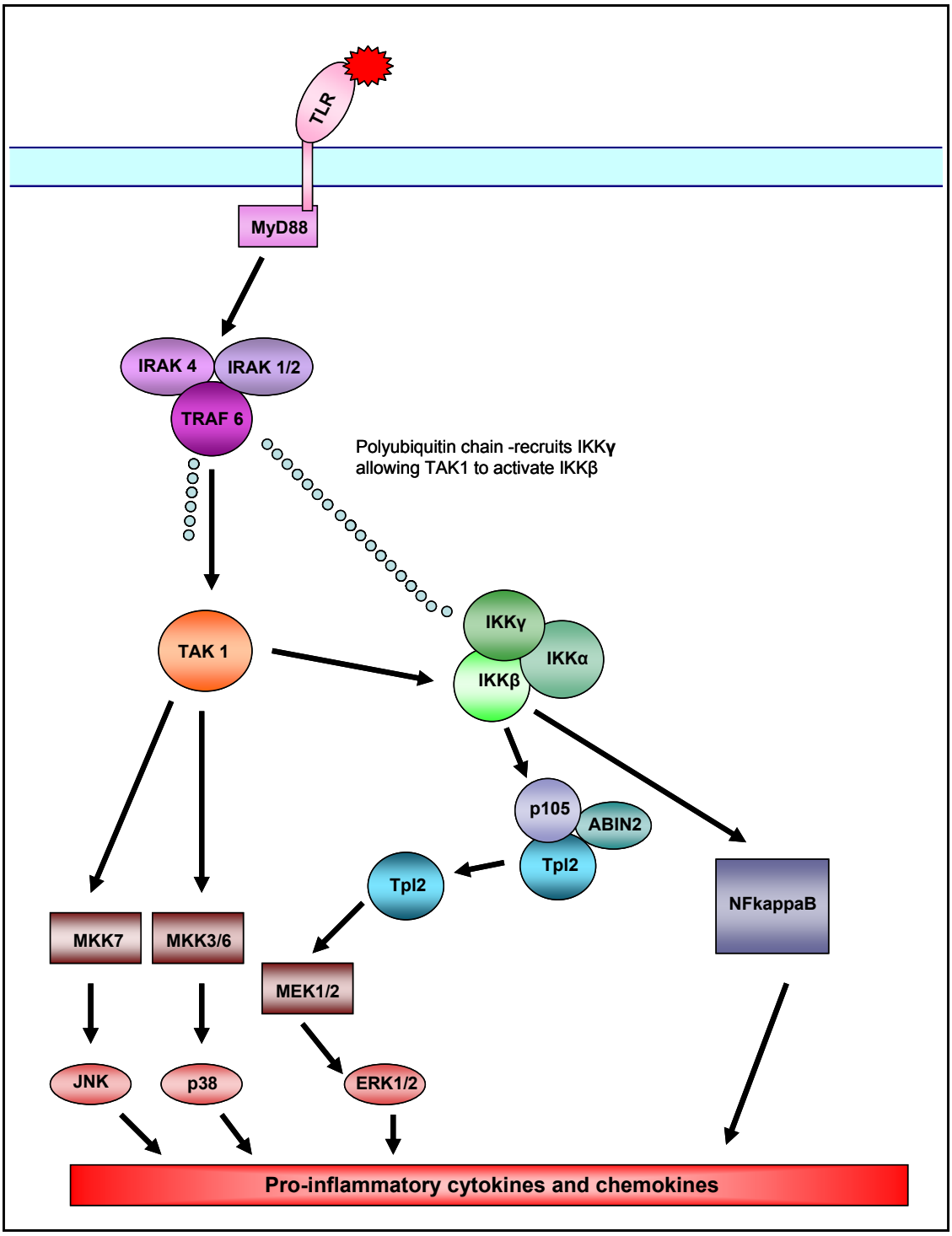
1.8.3.1 The MyD88 Dependent Pathway

After ligand binding to their TLRs, MyD88 recruits and activates the IL-1 receptor associated kinases IRAK4 and IRAK1/2, which then associate with the ubiquitin E3 ligase tumour necrosis factor receptor-associated factor 6 (TRAF6). It has been shown

that IRAK4 is absolutely required for activation of MAPKs and NFκB, whereas IRAK1 and 2 are only required for optimal activation of MAPKs and NFκB (Kawagoe 2008). Once IRAK-1 is active, it somehow activates TRAF6 E3 ligase, causing TRAF6 to then ubiquitinate itself and IKKγ (also known as Nemo). This creates a polyubiquitin scaffold bringing TAK1 into close proximity to the IKK complex, allowing TAK1 to activate the IKKβ, ultimately resulting in activation of NFκB (Bhjo and Chen 2009). TAK1 can also phosphorylate MKK3/6 and MKK4/7, resulting in the activation of p38 and JNK respectively (Figure 2).

TLR signalling also results in the activation of the MAPKs ERK1 and 2 via the MAP3K Tpl2. In unstimulated cells, Tpl2 is held in an inactive complex with ABIN2 and the NFκB subunit p105. Following IκB kinase (IKK) induced degredation of p105, active Tpl2 is released which then via MEK1/2 activates ERK1/2 (Waterfield, Zhang et al. 2003). It has been shown in Tpl2 knockout mice studies that Tpl2 is absolutely required for the activation of ERK1 and 2 by LPS, and that Tpl2/ERK activation are essential for TNFα induction in response to LPS (Dumitru, Ceci et al. 2000).

Figure 2 A Diagrammatic Representation of the TLR/MyD88 Dependent Signalling Pathways.



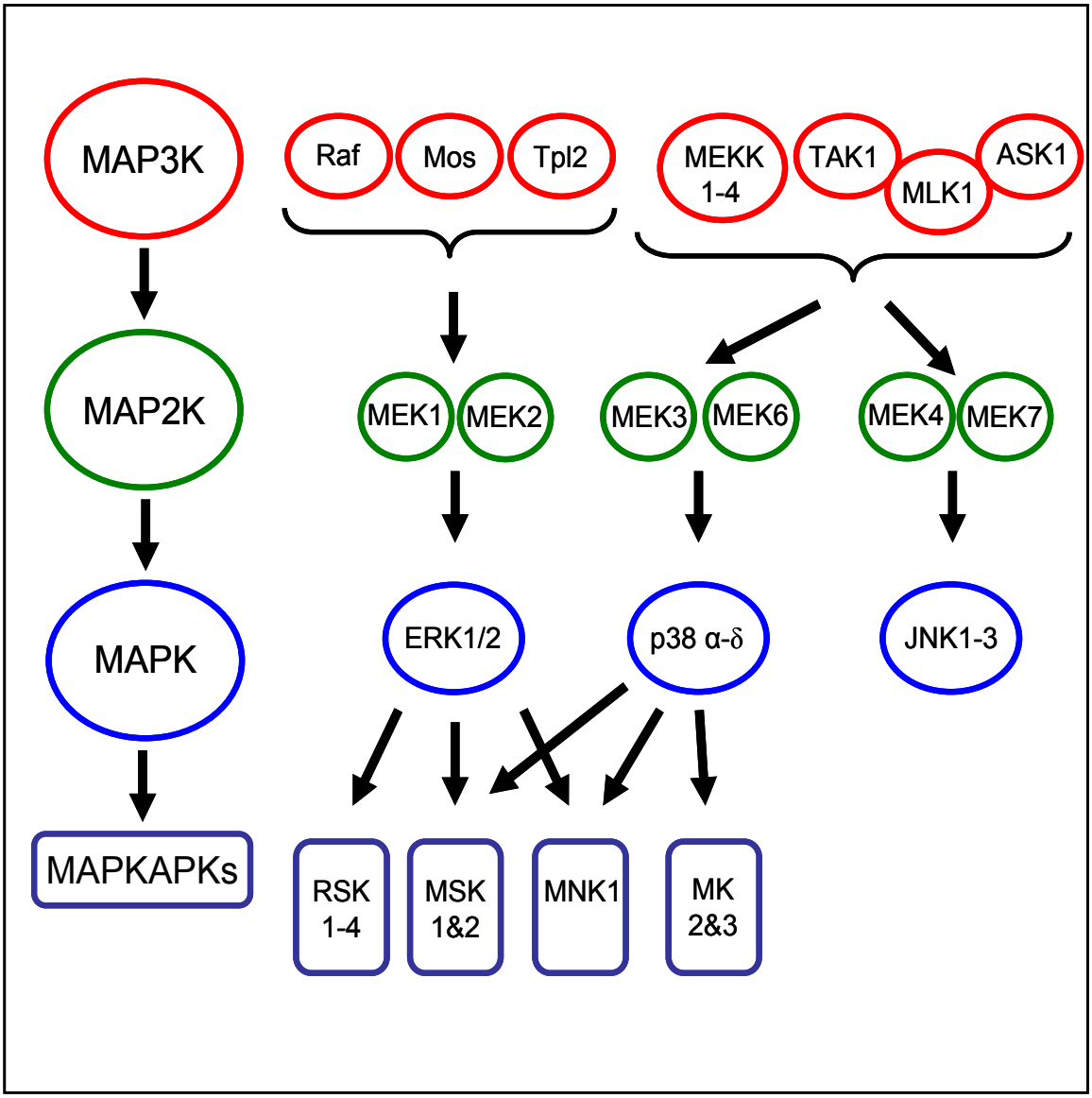
1.8.3.2 The TRIF Dependent Pathway

Interaction of TLR3 and TLR4 with TRIF leads to the generation of both a type I interferon response and inflammatory cytokines. Ligand binding to the TLR3 or TLR4 receptor causes recruitment of TRIF. Via its death domain, TRIF activates IKKε and TBK1 (Fitzgerald, McWhirter et al. 2003). This leads to phosphorylation of the transcription factor IRF3, which then induces IFNβ. In addition Trif can also activate the NFκB and MAPK signalling pathways (Yamamoto, Sato et al. 2002; Kawai and Akira 2008).

1.8.4 MAPK Signalling

In the immune system, MAPK signalling is pivotal in linking inflammatory stimuli to cellular outcome. MAPKs cascades consist of at least three tiers of kinases, MAPKs are activated by MAPK kinases (MAP2K), which are in turn activated by MAPK kinase kinase (MAP3K) that are activated by MAPK kinase kinase kinase (MAP4K) (figure3). This specificity allows the MAPK cascades to be involved in many diverse and specific cellular roles. The three major MAPK pathways are the classical MAPKs ERK1 and 2, p38 and JNKs (Raman, Chen et al. 2007).

Figure 3 Schematic Representation of the MAPK Cascades.
Adapted from (Roux and Blenis 2004)



1.8.5 Mitogen and Stress Activated Protein Kinases

Both Dectin-1 and TLR2 signalling result in the activation of the p38α and ERK1/2 MAPK pathways. This might be expected to lead to the activation of the mitogen and stress activated protein kinase (MSK) 1 and 2 (McCoy, Macdonald et al. 2007). MSKs are nuclear protein kinases that have been shown to be activated by both ERK1/2 and p38 in a variety of cell types. In macrophages MSKs have been shown to phosphorylate CREB, ATF1 and histone H3 in response to TLR agonists (Ananieva, Darragh et al. 2008), and as a result MSKs are able to regulate the transcription of specific immediate early genes. As discussed below, it is known that MSK1/2 can affect both pro-inflammatory and anti-inflammatory cytokine production.

1.8.5.1 MSK1/2 Knockout Mouse

Our laboratory has previously shown that when TLR4 is stimulated by LPS, mice lacking the MSK1/2 proteins show a significant increase in the production of pro-inflammatory cytokines TNFα, IL-6 and IL-12 relative to wild type (Ananieva, Darragh et al. 2008). There is no difference between wild type and knockout macrophages in their production of IL-1. However, the MSK1/2 knockout macrophages do show a decrease in their production of the pro-resolution factors IL-10 and DUSP1. It was shown that the lack of MSK1/2 prevents the activation of CREB and ATF1 which would, in normal circumstances, induce the transcription of DUSP1, a MAPK phosphatase which inactivates p38 and JNK. Via the phosphorylation of CREB, MSKs also increase IL-10 expression (Ananieva, Darragh et al. 2008). IL-10 is known to be a strong anti-

inflammatory cytokine which inhibits the production of pro-inflammatory cytokines by macrophages through the activation of the Janus kinase-STAT3 transcription factor signalling. IL-10 does not inhibit pro-inflammatory cytokines without STAT3 (Williams, Bradley et al. 2004), and in response to LPS the MSK1/2 knockout mice show not only much lower levels of IL-10, but much less phosphorylation of STAT3. This supports the theory that the excessive inflammatory response seen in the MSK1/2 knockout mice is due in part to the lack of IL-10 (Ananieva, Darragh et al. 2008).

In an attempt to characterise the *in vivo* response to TLR4 stimulation in the MSK1/2 deficient mice our laboratory previously took two approaches (Ananieva, Darragh et al. 2008). The first was to cause endotoxic shock in the mice through intraperitoneal injections of LPS. As expected, the MSK1/2 knockout mice were more sensitive, succumbing to endotoxic shock far more rapidly than their wild type counterparts. The second approach was to actually cause a bacterial infection by perforating the caecum and inducing peritonitis. Interestingly, in this setting of exposure to potentially virulent pathogens, the MSK1/2 knockout mice had a much higher survival rate than the wild type mice. It is suggested that the MSK1/2 knockout mice's massive pro-inflammatory cytokine response is protective in the setting of live pathogen infection, as the excessive inflammatory response is more effectively and rapidly destroying the pathogen, and once eliminated the inflammation can resolve.

1.9 IL-10 Signalling

IL-10 was first described as a cytokine produced by Th2 T helper cell clones that inhibited cytokine production by Th1 T helper cells (Fiorentino, Bond et al. 1989).The regulatory nature of IL-10 in controlling the macrophage inflammatory response was first reported when it was observed that IL-10 inhibited the production of pro-inflammatory cytokines IL-6, IL-1 and TNFα by LPS activated macrophages (Fiorentino, Zlotnik et al. 1991). Since then much research has focused on how exactly IL-10 exerts its anti-inflammatory effect, although the full mechanism has not yet been fathomed. In macrophages, it is understood that following TLR signalling IL-10 is secreted by macrophages, and that the secreted IL-10 feeds back, binding to the IL-10 receptor and activating the Janus kinase 1 (JAK1) (Rodig, Meraz et al. 1998), which in turn recruits and activates STAT3 (signal transducer and activator of transcription 3). The activation of STAT3 initiates the anti-inflammatory process, and it has been shown that STAT3 is essential for the repression of inflammation by IL-10 (Williams, Bradley et al. 2004). The mechanism of action of STAT3 is not fully understood, although it is thought not to act directly to reduce inflammatory cytokines, but instead to work via other, as yet unknown, genes.

The IL-10 knockout mouse clarifies the necessity of IL-10 in modulating and regulating the macrophage immune response. When challenged by bacterial pathogens IL-10 knockout mice mount massive inflammatory responses, producing huge unregulated amounts of IL-12 and TNFα (Gazzinelli, Wysocka et al. 1996), and are highly

susceptible to LPS induced toxic shock (Berg, Kuhn et al. 1995). Further, the IL-10 deficient mice can spontaneously develop chronic enterocolitis, which is though to be driven by resident gut flora (Kuhn, Lohler et al. 1993).

2. Aims

- Using a variety of ligands, inhibitors and genetically altered macrophages examine the differences between Dectin-1 and TLR signalling on cytokine production in macrophages.

- To examine the activation of ERK1/2 downstream of Dectin-1 in macrophages.

- To investigate the role of MSK1/2 in the response to fungal stimuli, whether via Dectin-1 or TLR2 activation, and the consequences on inflammation and cytokines in macrophages.

- To investigate the role of IL-10 in the suppression of pro-inflammatory cytokines in response to fungal stimuli in macrophages.

- To examine the differences between LPS and zymosan stimulation on macrophages.

3. Materials and Methods

3.1 Materials

All chemicals unless otherwise stated were obtained from Sigma or VWR. All solutions were prepared using deionised water (MilliQ system, Millipore) and where appropriate solutions were autoclaved at 120^{o}C, 15psi for 20mins.

3.2 Common Solutions

Phosphate Buffered Saline (PBS)	37mM NaCl 2.7mM KCl 4.3mM Na_2HPO_4 1.2mM KH_2PO_4
5x Sample Buffer	250mM Tris-HCL pH 6.8 32.5% (v/v) Glycerol 5% (w/v) SDS 5% (v/v) 2-β-mercaptoethanol
SDS Lysis Buffer	80% (v/v) Triton Lysis Buffer 10% (v/v) Glycerol 1% (w/v) SDS 0.1 % (v/v) 2- β-mercaptoethanol Bromophenol blue to colour
SDS-PAGE Running Buffer	25mM Tris 192mM Glycine 0.1% (w/v) SDS

SDS-PAGE Separating Gel	0.375M Tris-HCL pH8.6 10% or 12% (w/v) 29:1 Acrylamide:Bis- Acrylamide 0.01% (w/v) SDS 0.034% ammonium persulphate 0.08% (v/v) Temed
SDS-PAGE Stacking Gel	0.124M Tris-HCl pH6.8 3.73% (w/v) 29:1 Acrylamide:Bis-Acrylamide 0.01% (w/v) SDS 0.085% ammonium persulphate 0.1% (v/v) Temed
Transfer buffer	48mM Tris 39mM Glycine 20% (v/v) Methanol
Tris Buffered Saline and Tween (TBS-T)	50mM Tris pH 7.5 150mM NaCl 0.1% (v/v) Tween-20
Triton Lysis Buffer	50 mM Tris-HCl pH 7.5 1 mM EDTA 1mM EGTA 1% (v/v) Triton X-1000 1 mM Sodium Orthovanodate 50 mM Sodium Fluoride 5 mM Sodium Pyrophosphate 10 mM Sodium β-Glycerophosphate 0.27M Sucrose 0.1 % 2-β-Mercaptoethanol 1x protease inhibitor tablet (Roche)

3.3 Animals

MSK1/2 knockout mice and IL-10 knockout mice have been previously described (Kuhn, Lohler et al. 1993; Wiggin, Soloaga et al. 2002), and had been backcrossed onto a C57BL/6 background for at least 12 generations. CREB Ser133Ala knockin mice (described in (Wingate, Martin et al. 2009) were backcrossed onto a C57BL/6 background for at least 6 generations. Dectin-1 knockout femurs were obtained from Professor Gordon Brown (University of Aberdeen), and were on a 129/Sv genetic background (Taylor, Tsoni et al. 2007). All mice were maintained in specific pathogen free conditions, and in line with United Kingdom and European Union regulations. Work was approved by local ethical review and was carried out under the authority of a Home Office project license.

All routine animal care and biopsies were undertaken by the staff of the Transgenic Unit, University of Dundee. All mouse colony maintenance and breeding was organized by Dr. Arthur. All PCR genotyping of mouse biopsies was undertaken by Julia Carr (MRC Protein Phosphorylation Unit, University of Dundee).

3.4 Cell Culture

3.4.1 Derivation and Culture of BMDMs

Bone marrow derived macrophages (BMDMs) were derived from adult mice. After culling, the femurs were removed under sterile conditions. In a tissue culture hood the femurs were placed in bacteriological grade dishes and sterile PBS was used to flush the bone marrow from the femurs. The bone marrow suspension was then passed through a 100μm cell strainer into a 50ml falcon tube and centrifugated at 900 rpm for 5 minutes. The supernatant was aspirated and the pellet resuspended in 20ml of media (DMEM (Gibco) with 10% FBS (Biosera, heat inactivated), 5mg/ml L-Glutamine (Gibco), 5% antibiotic-antimycotic (Gibco) and 5ng/ml Macrophage Colony Stimulating Factor (R&D Systems). The media/cell suspension was then divided between 2 x 10cm bacteriological grade culture plates. The cells were then incubated at 37°C in 5% CO_2 for seven days.

On day seven, the dishes containing the macrophages are removed from incubator and the media aspirated. Each dish was washed with 5ml of sterile PBS. 5ml of versene (Gibco) was added, and the cells incubated for 10 minutes to loosen cell adhesions. The cells were then removed with a sterile cell scraper and centrifuged at 1000 rpm for 5 minutes. The supernatant was aspirated and the pellet resuspended in 12ml of media (as above). This was then divided into 2ml per well of a 6 well plate, or 1ml per well of a 12 well plate dependent on the experimental use of the BMDMs (cell density $2x10^5$ cells/ml). The cells were incubated overnight and stimulated the following day.

3.4.2 RAW264.7 Macrophage-Like Cell Line

RAW264.7 cells are a mouse macrophage cell line obtained from the American Type Culture Collection (Manassas, U.S.A.). They are an adherent cell line that were maintained in DMEM (Gibco) supplemented with 10% FBS (Sigma, heat inactivated), 5% L-Glutamine (Gibco) and 5% penicillin-streptomycin (Gibco). They were maintained in an incubator at 37°C in 5% CO_2. The day prior to use, the cells were scrapped into the media and then spun down at 1000 rpm for 5 minutes. The cells were then resuspended in fresh media and plated onto 6 or 12 well plates. The cells were incubated overnight and stimulated the following day.

3.4.3 A20 B-cell Lymphoma Mouse Cell Line

The A20 cell line is a mature mouse B-cell lymphoma cell line that expresses a mature B-cell receptor. We were kindly donated the cells by Dr Sharon Matthews, University of Dundee. The A20 cells are semi-adherent and as such are maintained at a density of between $1x10^5$ and $1x10^6$ cells per ml in RMPI 1640 media (Gibco), supplemented with 10% heat inactivated FBS (Sigma, heat inactivated), 5% L-Glutamine (Gibco), 5% penicillin-streptomycin (Gibco) and 50μM 2-β-mercaptoethanol, and were incubated at 37°C in 5% CO_2. The day prior to use, the adherent cells were scrapped into the media and then spun down at 1000 rpm for 5 minutes. The cells were then resuspended in fresh media and plated onto 10cm plates. The cells were incubated overnight and stimulated the following day.

3.5 Stimulation and Inhibition of Cells

3.5.1 BMDM Stimulation

The BMDMs were stimulated with a range of microbial ligands, as indicated in the figure legends. These were zymosan (Sigma) used at 200µg/ml, depleted zymosan (Invivogen) used at 200µg/ml and curdlan (Sigma) used at 10µg/ml. LPS (from *E. Coli* 055:B5, Sigma) was used at 100ng/ml, Pam_3CSK_4 (Invivogen) was used at 1µg/ml, Poly(I:C) (Invivogen) was used at 10 µg/ml and CpG (Invivogen) was used at 2µM. Recombinant mouse IL-10 (R&D Systems) was used at 100ng/ml. In some experiments, BMDMs were treated with beads to simulate phagocytosis of fungal particles; these were Estapor microsphere 0.8µm latex calibrated particles from Merck.

3.5.2 A20 Cell Stimulation

The A20 cells were stimulated via their B-cell receptor, to ensure SYK dependent signalling pathway activation. For this I used $F(ab')_2$ fragment rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a concentration of 10µg/ml to cross-link the BCR and simulate antigen stimulation.

3.5.3 Inhibitors

The BMDMs were inhibited with a range of small molecule inhibitors. The MEK inhibitor PD184352 and the p38 inhibitor SB203580 were used at 2µM and 5µM respectively. The Tpl2 inhibitor Compound 1 (also known as SHN681) was used at

10μM. The IKK inhibitor BIX02514 was used at 10μM. The COX2 inhibitor NS398 was used at 10μM. All of these inhibitors were from the DSTT, University of Dundee, either being synthesised by the team, or purchased from Calbiochem. These concentrations all correspond to those previously established in the laboratory as being the minimum required to inhibit the target kinase in cells.

The SYK inhibitors I, II, III and IV were all purchased from Calbiochem. R406 was purchased from American Custom Chemical Corporation. Piceatannol was purchased from both Sigma and Calbiochem. The SYK inhibitors were all used at varying concentrations as stated.

3.5.4 Neutralising Antibodies

The Dectin-1 neutralising antibody was a rat IgG monoclonal antibody to murine dectin-1 (Invivogen, clone 2A11) and was used at 10μg/ml. Isotype rat antibody (R&D systems) was used at 10μg/ml as a control.

3.6 Lysis of Cells for RNA

After stimulation of cells, the media was removed and frozen at -80ºC for later cytokine analysis. The remainder of the media was aspirated. The cells were then washed with sterile PBS and 350µl of Qiagen RLT buffer with 1% β-mercaptoethanol was then added per well. The cells were disrupted with a cell scraper and then transferred to a Qiagen QIAshredder column. The lysate column was then centrifuged at 13000 rpm for 2 minutes. The column and lysate were then frozen to store at -80 ºC. When required, the lysate were defrosted and total RNA was extracted using the Qiagen RNeasy micro kit following the manufacturer's instructions. The RNA was eluted in 14µl of RNase free water and stored at -80 $^{\circ}$C.

3.7 Determination of RNA Concentration and Reverse Transcription

1.4µl of purified RNA was quantified using the nanodrop machine (Thermo Scientific), as per the manufacturer's instructions. Absorbance at 260nm was read, and the sample concentration determined by the nanodrop software. 0.7µg of RNA was then diluted into 10µl of nuclease free water, and added to 4µl of iscript reaction mix (Bio-Rad) and 1µl of iscript reverse transcriptase (Bio-Rad). The samples were then reverse transcribed by incubating at 25ºC for 5 min, 42ºC for 30 min and finally 85ºC for 5 min. 200µl of nuclease free water was added to the cDNA. The cDNA was frozen at -20ºC until required.

3.8 Quantitative PCR

3.8.1 Methodology

Quantitative PCR was performed in 96 or 384 well plates (Bio-Rad) using SYBR green based detection system (Bio-Rad SsoFast EvaGreen supermix) with a Bio-Rad CXF 96 or Bio-Rad CXF 384 thermal cycler. Each reaction comprised 10µl SYBER green supermix, 3.4µl nuclease free water, 0.8µl 10µM sense primer, 0.8µl 10µM antisense primer and 5µl of cDNA. The plate was then sealed with optical tape (Bio-Rad), and cycled 95°C for 30 sec to initially denature, followed by 40 cycles at 60°C for 25 sec, 95°C for 1 min, then 65°C for 1 min. Each sample was performed in duplicate. 18s ribosomal RNA values were used for normalisation. The relative mRNA levels were calculated using the following equation:

$$\text{Relative mRNA level} = \frac{E_u^{(ct\ uc - ct\ us)}}{E_r^{(ct\ rc - ct\ rs)}}$$

Where E is efficiency of PCR, Ct is the threshold cycle, u is the mRNA of interest, r is the 18s ribosomal RNA, s is the sample and c is the unstimulated control.

3.8.2 Primers

All primers were designed using the Beacon Designer 7.0 software programme and custom synthesised by Invitrogen. The primers were reconstituted in nuclease free water

at a concentration of 100μM, and then diluted to a working solution of 10μM as required. All primers were stored at -20^{0}C and aliquots of working stock were defrosted to room temperature prior to use. Amplification of the correct product was confirmed by members of our laboratory through cloning and sequencing of the PCR product. The sequences for all primers used are shown in table 2.

3.9 Multiple Cytokine Secretion Assay

Following cell stimulation for mRNA analysis, the media from the cells was stored at -80°C. When appropriate, the media was thawed and cytokine concentrations in the cell media were detected using the Luminex-based Bio-plex mouse cytokine 5-Plex and 9-Plex panels (Bio-Rad) as per manufacturer's instructions.

Table 2 Primer Sequences Used for Quantitative PCR

Primer Name	Sense Primer	Anti-sense Primer
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
c-fos	CTACTGTGTTCCTGGCAATAGC	AACATTGACGCTGAAGGACTAC
c-jun	CGCCTCGTTCCTCCAGTC	ACGTGAGAAGGTCCGAGTTC
COX2	AATATCAGGTCATTGGTGGAGAGG	TCAGACCAGGCACCAGACC
Dusp-1	CCACAGGACACCGCACAAG	AGCGAAGAAGGAGCGACAATC
EBI-3	GCCGCTCCCCTGGTTA	CAATGAAGGACGTGGATCTGGTC
Egr-1	ACAGAAGGACAAGAAAGCAGAC	CCAGGAGAGGAGTAGGAAGTG
Egr-3	TGTGATGGACATCGGTCTGAC	GGCACCAGTTGGAAGGAGAG
Fos-b	CCAGGGTCAACATCCGCTAAG	GGTGAGGACAAACGAGGAAGTG
G-CSF	TGCACCCTGACTGGAGTTAC	TGAAATCTCGATGTGTCCACAG
GFI-1	GGTCCCAAACACTGATG	CAACTGGTCCGTTATCC
GFI-1 unspliced	TTGTAGGAGATGCTTGAA	TTGACATAGAGGAAGATACT
GFI-1b	TCTAGTGAAGAGTAAGAAGG	CTGTTTGATTGTGTTCCA
GM-CSF	CTACTGATAGGGACCATTA	GGTAAGACATTCTCAATAAATAG
Interferon β	GGAAAAGCAAGAGGAAAGATTGAC	CCACCATCCAGGCGTAGC
Interferon γ	AGCCAAGACTGTGATTGC	TTATTGGTCAGTGAAGTAAAGG
IL-1a	TCAAGCAACGGGAAGATTC	TATCTCAAATCACTCTGGTAGG
IL-1b	GACGGACCCCAAAAGATGAAGG	GTGATACTGCCTGCCTGAAGC
IL-1ra (total)	TTACAAGGACCAAATATCAAACTAGAAG	GGATGCCCAAGAACACACTATG
IL-1ra (cytoplasmic)	TCCTTTATACACAGCAAGTCTC	TTCTGAAGGCTTGCATCTTG

IL-1ra (secretory)	AGTCGCTAGTCTCTATTGCC	TTCTGAAGGCTTGCATCTTG
IL-2	CCCAAGCAGGCCACAGAATTG	CTCATCATCGAATTGGCACTCAAATG
IL-4	CTTGATAAACTTAATTGTCTCTC	AATGCCGATGATCTCTC
IL-6	TTCCATCCAGTTGCCTTCTTG	AGGTCTGTTGGGAGTGGTATC
IL-10	CCCTTTGCTATGGTGTCCTTTC	GATCTCCCTGGTTTCTCTTCCC
IL-12a	TATCTCTATGGTCAGCGTTCC	TGGTCTTCAGCAGGTTTCG
IL-12b	TCATCAGGGACATCATCAAACC	TGAGGGAGAAGTAGGAATGGG
IL-13	ACAAAGCAACTGTTTCG	AGTCTGATGTGAGAAAGG
IL-23A (p19)	ATCCAGTGTGAAGATGGTTGTGAC	TCCTAGTAGGGAGGTGTGAAGTTG
IL-27	GAGGAGGACAAGGAGGAAGAGG	GGGAGTGAAGGAGCTGGTAGC
Light	CTGCATCAACGTCTTGGAGA	GATACGTCAAGCCCCTCAAG
Mir132	CGGTGACTCAGCCTAGATGG	GGACGGGACAGGGAAGGG
Nor1	GCCATCTCCTCCGATCTGTATG	GAGGCCGTCAGAAGGTTGTAG
Nur1	GAAGAGAGCGGACAAGGAGATC	AAGGCATGGCTTCAGCAGAG
Nur77	CCTGTTGCTAGAGTCTGCCTTC	CAATCCAATCACCAAAGCCACG
SBNO2	AATGCCTCTGTCTTCTC	GTGACTGATAGCTGATGA
SerpinB2	CTGGATGAAGATGATGTTGTGGTC	GAAGAATGGACTTGAGTTCGTAGC
SPHK1	ACAGCAGTGTGCAGTTGATGA	GGCAGTCATGTCCGGTGATG
SOCS3	AGAAGATTCCGCTGGTACTGAG	AAAGATGCTGGAGGGTGGC
Jun-b	TGCACGAAAATGGAACAGCC	GGTGGGTTTCAGGAGTTTGTAG
NFIL-3	TTCAGGACTACCAGACAT	TGCTTGATGACTGAGATG
TNFα	CAGACCCTCACACTCAGATCATC	GGCTACAGGCTTGTCACTCG

3.10 Detection of Protein by Immunoblotting

3.10.1 Lysis of Cells for Protein for Immunoblotting

After stimulation of cells, the media was aspirated. The cells were then washed with sterile PBS. 300µl of SDS lysis buffer was added to each well. The samples were heated to 97°C for 10 minutes, and then passed repeatedly through a 25 gauge needle to homogenise the sample. Samples were frozen at -20°C until required.

3.10.2 Resolution of Protein Samples on SDS-Polyacrylamide Gel Electrophoresis (PAGE)

For western blotting, 30µl of sample and 4µl of molecular weight standard marker (Bio-Rad) were loaded on the SDS-PAGE gel. Self poured Tris-glycine gels were used with either 10% or 12% acrylamide with tris-glycine running buffer. The gels were then electrophoresed at 100V for 150-220 minutes. Proteins were then transferred onto nitrocellulose membrane.

3.10.3 Transfer of Proteins onto a Nitrocellulose Membrane

Bio-Rad transfer modules were used to transfer proteins from the SDS-PAGE gel onto the nitrocellulose membrane. All materials were pre-soaked in transfer buffer prior to assembly of transfer cassette. The transfer cassette comprised of 2 sponges, one 7.5cm by 9cm 3mm filter paper, the SDS page gel, one 7.5cm by 8.5cm nitrocellulose membrane fully covering the gel, followed by a second piece of 3mm filter paper, and a further

sponge. The cassette was placed into a Bio-Rad tank, and the process repeated for a second gel and cassette. The tank was then filled to the top with transfer buffer, and an ice pack was added. The proteins were then transferred at 100V for 1hour.

3.10.4 Western Blotting

Following the transfer of proteins onto a nitrocellulose membrane, the membranes were then blocked with 5% dried milk/ tris-buffered saline with 0.1% tween (TBS-T) for 45 minutes at room temperature on a shaker. The membranes were incubated with primary antibody diluted 1:1000 in either 5% dried milk/TBS-T or 5% bovine serum albumin/TBS-T overnight at 4^{o}C. Table 3 shows a full list of all antibodies used. Unbound primary antibody was then removed by washing the membrane with TBS-T for 3 x 10 minutes. Following this, the membranes were incubated in the appropriate horseradish conjugated secondary antibody (Pierce) diluted 1:2000 in 5% dried milk/TBS-T for 1 hour at room temperature. Unbound secondary antibody was removed by washing the membrane with TBS-T for 3 x 10 minutes. The signal was detected with enhanced chemiluminescence reagent (GE Healthcare).

Table 3 Antibodies Used for Western Blotting

Antibody	Isotype	Company	Order Number
CREB	Rabbit IgG	Cell Signaling Technology	#9197
Phospho-CREB (Ser133)	Mouse IgG1κ	Millipore	05-667
ERK1/2	Rabbit Polyclonal	Cell Signaling Technology	#9102
Phospho-ERK1/2 (Thr202/Tyr204)	Rabbit Polyclonal	Cell Signaling Technology	#9101
GAPDH	Rabbit IgG	Cell Signaling Technology	#2118
GFI-1	Rabbit Polyclonal	Santa Cruz Biotechnology	Sc-22796
GFI-1B	Goat Polyclonal	Santa Cruz Biotechnology	Sc-8559
IκBα (Amino-terminal Antigen)	Mouse IgG1	Cell Signaling Technology	#4814
IRF-3	Rabbit IgG	Cell Signaling Technology	#4302
Phospho-IRF3 (Ser396)	Rabbit IgG	Cell Signaling Technology	#4947
JNK	Rabbit Polyclonal	Cell Signaling Technology	#9258
Phospho-JNK (Thr183/Tyr185)	Rabbit Polyclonal	Cell Signaling Technology	#9251
Phospho-MAPKAPK-2 (Thr334)	Rabbit Polyclonal	Cell Signaling Technology	#3041
Phospho-MAPKAPK-2	Rabbit Polyclonal	Cell Signaling Technology	#3044
MEK1/2	Rabbit Polyclonal	Cell Signaling Technology	#9122
Phospho-MEK1/2 (Ser217/221)	Rabbit Polyclonal	Cell Signaling Technology	#9121
MSK1	Sheep Polyclonal	DSTT, University of Dundee	N/A
Phospho-MSK1 (Thr581)	Rabbit Polyclonal	Cell Signaling Technology	#9595
Phospho-MSK1 (Ser376)	Rabbit Polyclonal	Cell Signaling Technology	#9591
MyD88	Rabbit polyclonal	Cell Signaling Technology	#3699
Phospho-NF-κB p65 (Ser276)	Rabbit polyclonal	Cell Signaling Technology	#3037

p38 MAPK	Rabbit polyclonal	Cell Signaling Technology	#9212
Phospho-p38 MAPK (Thr180/Tyr182)	Rabbit polyclonal	Cell Signaling Technology	#9211
Phospho-p90RSK (ser380)	Rabbit polyclonal	Cell Signaling Technology	#9341
STAT3	Rabbit polyclonal	Cell Signaling Technology	#9132
Phospho-STAT1 (Tyr701)	Rabbit polyclonal	Cell Signaling Technology	#9171
Phospho-STAT3 (Ser727)	Rabbit polyclonal	Cell Signaling Technology	#9134
Phospho-STAT3 (Tyr705)	Rabbit polyclonal	Cell Signaling Technology	#9131
Phospho-STAT5 (Tyr694)	Rabbit IgG	Cell Signaling Technology	#9359
Phospho-STAT6 (Tyr641)	Goat IgG	Santa Cruz Biotechnology	Sc-11762
SYK	Rabbit polyclonal	Cell Signaling Technology	#2712
Phospho-SYK (Tyr323)	Rabbit polyclonal	Cell Signaling Technology	#2715
Phospho-SYK (Tyr525/526)	Rabbit polyclonal	Cell Signaling Technology	#2711
Phospho-SYK (Tyr352) /Phospho-ZAP-70 (Tyr319)	Rabbit polyclonal	Cell Signaling Technology	#2701
TBK1	Rabbit IgG	Cell Signaling Technology	#3504
Phospho-TBK1 (Ser172)	Rabbit IgG	Cell Signaling Technology	#5483
Tpl2	Rabbit polyclonal	Santa Cruz Biotechnology	Sc-720

4. Results

4.1 Dectin-1 Stimulation Activates MAPK Signalling Leading to MSK Activation and CREB Phosphorylation

Dectin-1 is an important PRR for fungal pathogens. In order to study Dectin-1 signalling I made use of several ligands thought to act via either Dectin-1 alone or a combination of Dectin-1 and TLRs. Initially, I looked at the effect of fungal ligands on the signalling cascades in wild type BMDMs to ascertain which pathways were activated by fungal stimulation. To determine activation of a particular kinase, I used Western blotting to look at the phosphorylation status of the site known to be responsible for the activation of the kinase. For the MAPKs p38, ERK1/2 and JNK this was the TXY motif, which when phosphorylated causes the kinase to become active. For MSK1 Thr581 was used. This residue is phosphorylated by ERK1/2 or p38, resulting in activation of the C-terminal kinase domain in MSK1. This allows the C-terminal domain to phosphorylate and activate the N-terminal kinase domain which is responsible for the phosphorylation of substrates. I also looked at the phosphorylation of the transcription factors CREB and ATF1 which are substrates of MSK1.

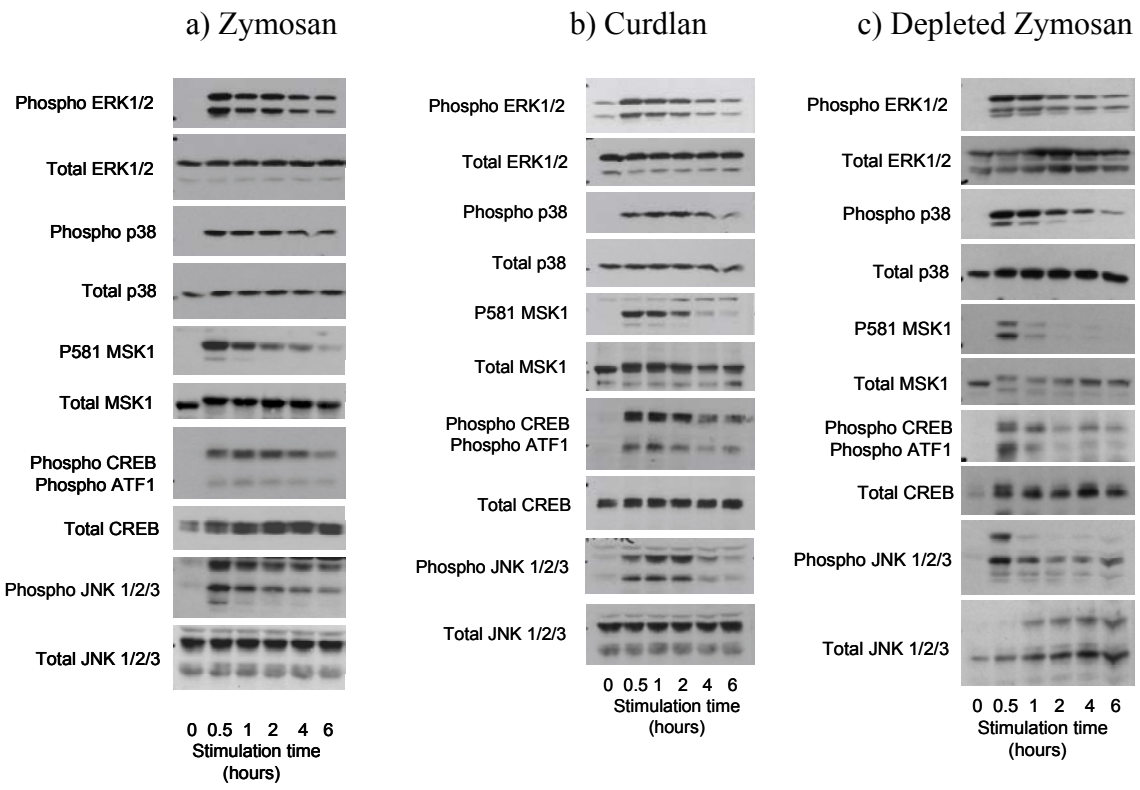
Zymosan is extracted from yeast cell wall and, based on the literature, I expected it to activate both TLR2 and Dectin-1 (Ikeda, Adachi et al. 2008). Wild type BMDMs were stimulated with zymosan alone, over a period of 6 hours, and Figure 4a shows that the three major MAPK pathways, ERK1/2, p38 and JNK were all activated by zymosan. In

addition, activation of MSK1 and phosphorylation of its substrates CREB and ATF1 were also observed.

As zymosan stimulates both Dectin-1 and TLR2, the effects of depleted zymosan and curdlan, which are reported to be Dectin-1 specific, were examined (Figure 4b&c). This shows that both these stimuli also activated the classical MAPK pathways as well as MSK1 and CREB. Based on multiple experiments, zymosan was a stronger stimulus for MAPK signalling relative to either depleted zymosan or curdlan (data not shown).

Figure 4 Fungal Stimulation of Wild Type BMDMs.

BMDMs were stimulated with a) 200µg/ml zymosan b) 10µg/ml curdlan c) 200µg/ml depleted zymosan and incubated for the stated times. Cells were lysed in 1% SDS lysis buffer and levels of the indicated proteins determined by immunoblotting.



4.2 The Examination of Dectin-1 Ligand Specificity

To clarify whether depleted zymosan and curdlan only stimulate through Dectin-1 in BMDMs, a Dectin-1 neutralising antibody was used prior to stimulation. Figure 5 shows that the neutralising antibody prevented the activation of ERK1/2 and p38 by depleted zymosan, however, curdlan still activated these pathways despite the presence of the neutralising antibody.

To confirm these results, BMDMs from Dectin-1 knockout mice were tested (Figure 6). Zymosan is still able to activate signalling pathways in the knockout BMDMs, as shown by the activation of p38 and ERK1/2, but this was reduced relative to the activation seen in wild type BMDMs. As NFκB is important in regulating cytokine production I also looked at phosphorylation of the p105 NFκB subunit. This is a substrate of the IKK complex and thus provides an indication of the activation of the canonical NFκB pathway, in addition to being required for Tpl2 activation. Like MAPK signalling, p105 phosphorylation in response to zymosan was reduced in the Dectin-1 knockout. This is expected as zymosan can also stimulate TLR2 in addition to Dectin-1. Depleted zymosan was unable to activate MAPK or p105 signalling in the Dectin-1 knockout BMDMs, which is consistent with what was seen with the neutralising antibody. In line with this, MSK1 activation and CREB phosphorylation were also blocked. Curdlan was also unable to activate MAPK or p105 signalling pathways in the Dectin-1 knockout. This is in contrast to the neutralising antibody results that would suggest that curdlan should still signal in the Dectin-1 knockout. This maybe explained by the possibility that the Dectin-

1 knockout is missing another crucial receptor, other than Dectin-1, that is required for, or contributes to, curdlan signalling as curdlan is a very crude ligand preparation. It is also possible that there is a flaw in the neutralising antibody experiments, for example a component of the curdlan solution may cause dissociation of the antibody from the receptor allowing Dectin-1 to be available for curdlan binding and activation of the receptor.

Based on the Dectin-1 knockout, depleted zymosan and curdlan should not signal via TLR2. To confirm this I used BMDMs from MyD88 knockout mice, which are deficient in all MyD88 dependent TLR signalling (that is all TLRs with the exception of TLRs 3 and 4). In line with this, figure 7 shows that the TLR2 agonist Pam3CSK4 did not induce p38, ERK1/2 or p105 phosphorylation in the MyD88 knockout macrophages. Curdlan, depleted zymosan and zymosan were all able to activate p38 and ERK1/2, as well as inducing p105 phosphorylation in the MyD88 knockout BMDMs. This is consistent with the activation of these pathways by Dectin-1, which does not associate with MyD88, by these stimuli. Interestingly however, curdlan activation of p38, ERK1/2 and p105 phosphorylation in the MyD88 knockout BMDMs does appear to be reduced slightly when compared to the wild type. This implies that curdlan may also be able to signal independently of Dectin-1, but that this contribution is minimal, explaining why it was not obvious in the Dectin-1 knockout experiments. This finding also aids in the explanation of the neutralising antibody data, and is supported by very recent further work in our laboratory, which has shown that when curdlan is used at very high concentrations (200μg/ml), it can activate these signalling pathways independently of Dectin-1 (data not shown).

Figure 5 Wild Type BMDMs Treated with Dectin-1 Neutralising Antibody, then Stimulated with Dectin-1 Ligands.

BMDMs were pre-incubated for 1 hour with Dectin-1 neutralising antibody (10µg/ml) or isotype antibody (10µg/ml). BMDMs were then stimulated with either 200µg/ml of depleted zymosan or 10µg/ml of curdlan. BMDMs were incubated with the stimuli for 30 minutes; cells were then lysed in 1% SDS-lysis buffer and the levels of the indicated proteins were determined by immunoblotting.

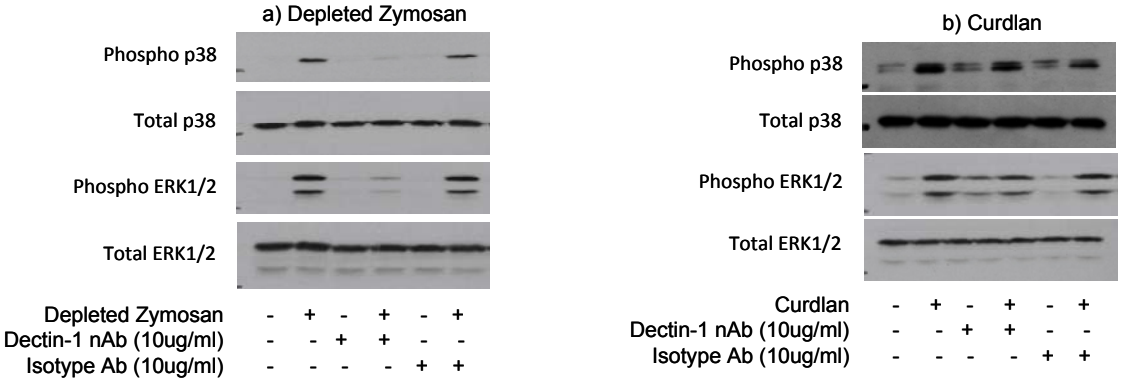


Figure 6 Dectin-1 Wild Type and Knockout BMDMs.

BMDMs stimulated with a) 200µg/ml zymosan b) 10µg/ml curdlan c) 200µg/ml depleted zymosan for the times stated. Cells were then lysed in 1% SDS-lysis buffer. The levels of the indicated proteins were determined by immunoblotting.

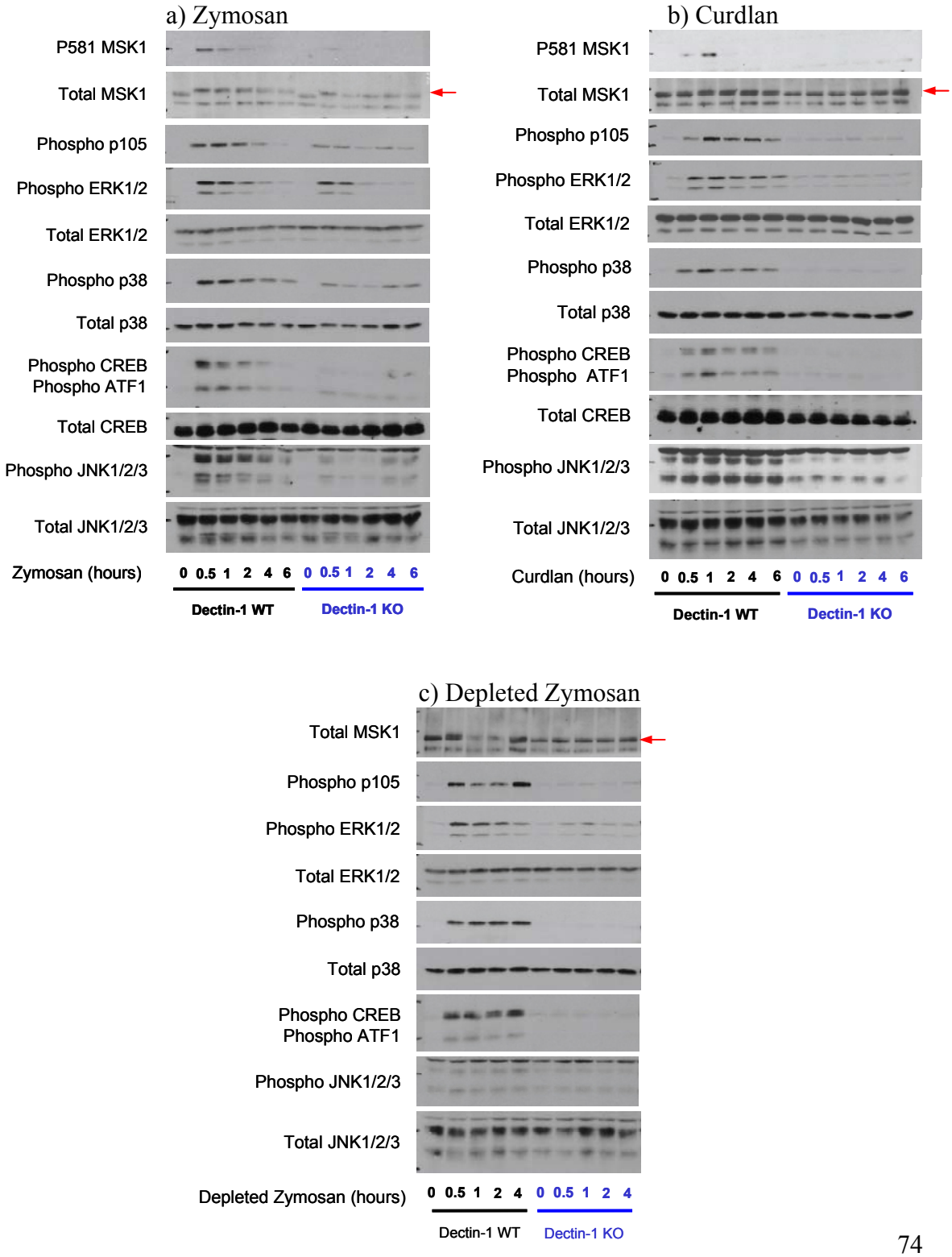
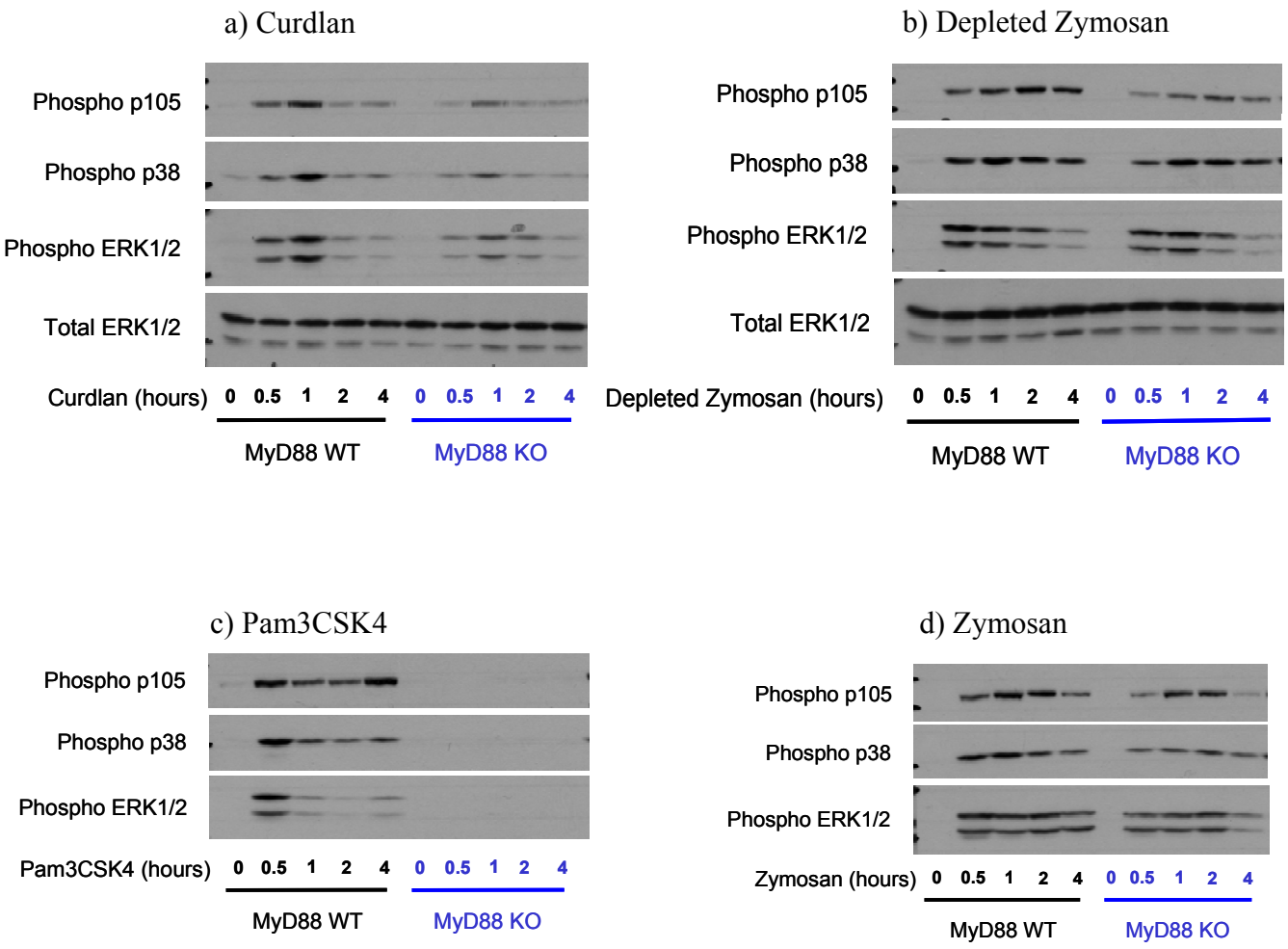


Figure 7 MyD88 Wild Type and Knockout BMDMs.

BMDMs were stimulated with a) 10µg/ml curdlan b) 200µg/ml depleted zymosan c) 1µg/ml Pam3CSK4 or d) 200µg/ml zymosan for the times stated. Cells were then lysed in 1% SDS-lysis buffer. The levels of the indicated proteins were determined by immunoblotting.



4.3 SYK Inhibition

It has been shown that Dectin-1 requires SYK for activation of downstream signalling pathways and appropriate cytokine responses to fungal ligands. One key study by Rogers *et al* showed that SYK directly interacts with the Dectin-1 tail and that phosphorylated SYK is associated with zymosan containing phagosomes. Further, through the use of SYK deficient DCs they showed that in the absence of SYK, DCs are unable to produce IL-2 and IL-10 in response to zymosan (Rogers, Slack et al. 2005). SYK inhibitors can therefore provide a useful tool to analyse Dectin-1 function.

Several small molecules have been described as SYK inhibitors, however in most cases selectivity has not been determined. The most commercially available SYK inhibitors are SYK inhibitor I, SYK inhibitor II, SYK inhibitor III, SYK inhibitor IV, R406 and piceatannol. The structures of these inhibitors and their IC_{50} for SYK are shown in Table 4. As there is only limited information on specificity for these compounds, their ability to inhibit the activity of a panel of kinases *in vitro* was determined by the International Centre for Kinase Profiling within the DSTT, University of Dundee. The results of this screen are shown in figure 8. This shows that SYK inhibitor I at 1μM was able to decrease SYK activity to 19%, although unfortunately inhibited other kinases involved in cytokine production (such as IKKe) to a much greater extent. SYK inhibitor II at 1μM was very selective and inhibited SYK by a significantly greater percentage than almost any other kinase tested in the panel. SYK inhibitor III at 1μM was a very poor inhibitor of SYK in the screening experiments, actually causing an increase in SYK activity. SYK inhibitor IV at 1μM reduced SYK activity to 32%, but inhibited a large number of

kinases in the panel to the same extent. R406 at 1μM reduced SYK activity to 5%, but notably also inhibited TBK1 and Src to similar levels. Piceatannol at 10μM reduced SYK activity to 66%, but reduced the activity of many more kinases by a greater level than this. Notable examples of relevant kinases inhibited more than SYK by piceatannol include ERK1, IKKe, PKD1, AMPK and IGF1.

Table 4 The Structures and SYK IC_{50} of the 6 Most Commercially Available SYK Inhibitors.

IC_{50} values were obtained from the relevant manufacturer. As the IC_{50}s were not all performed and analysed at the same time, we must consider that differences in the assay, such as ATP concentration, makes it difficult to directly compare values.

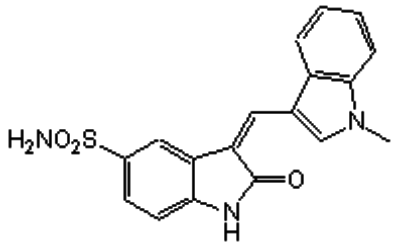
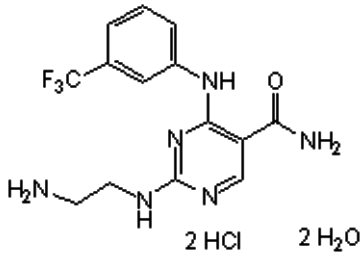
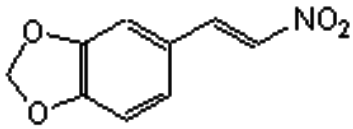
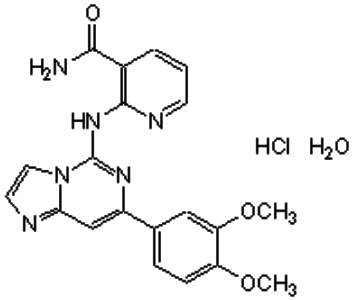
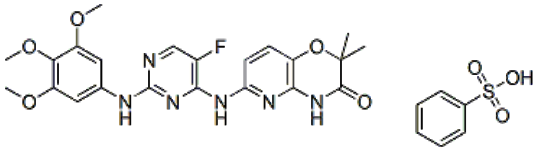
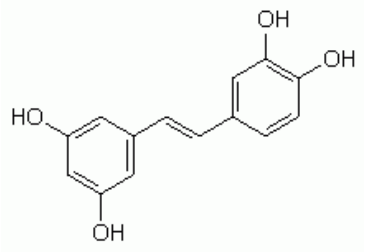
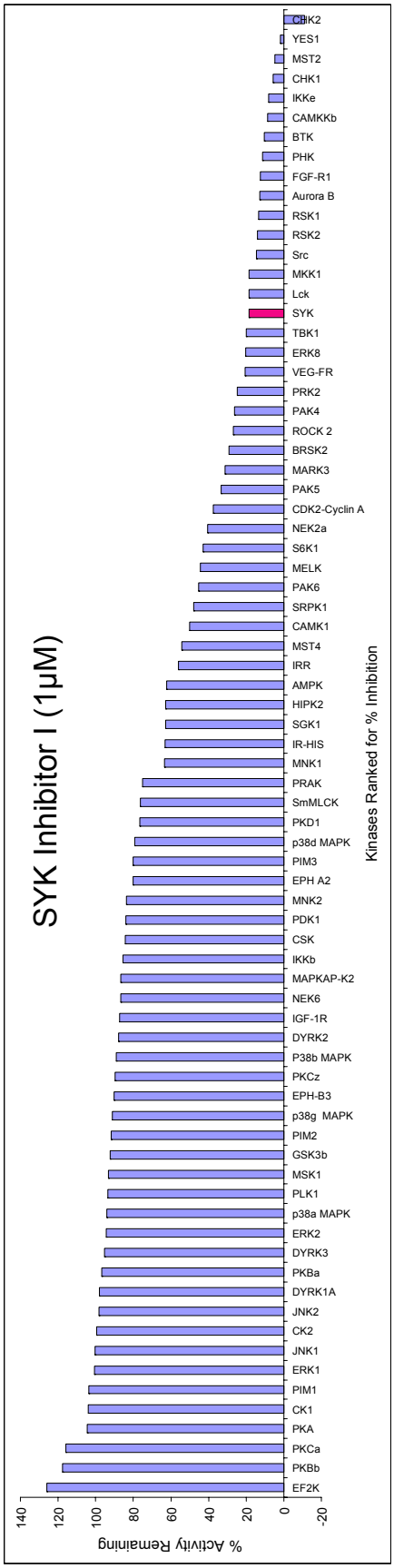
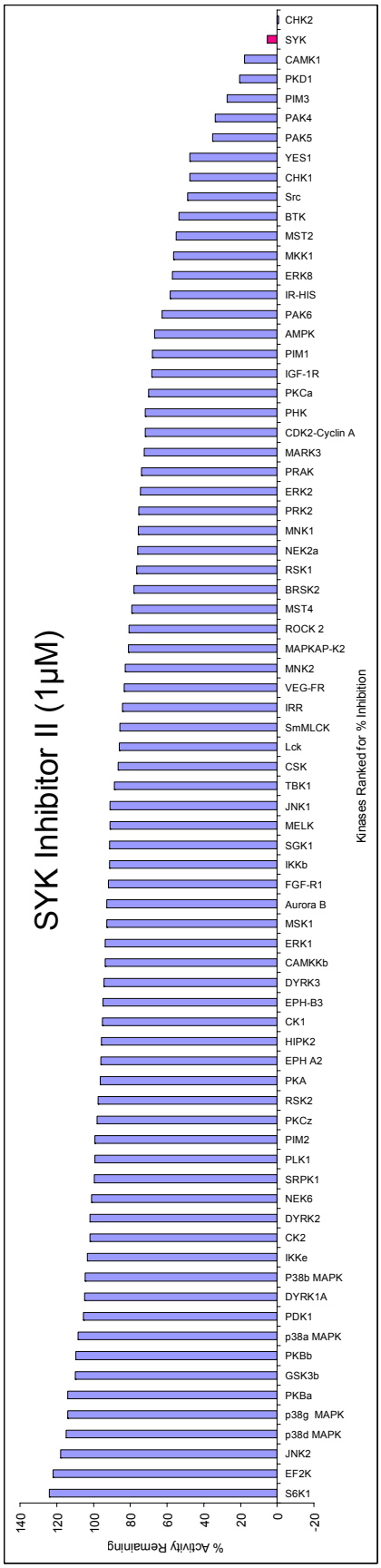
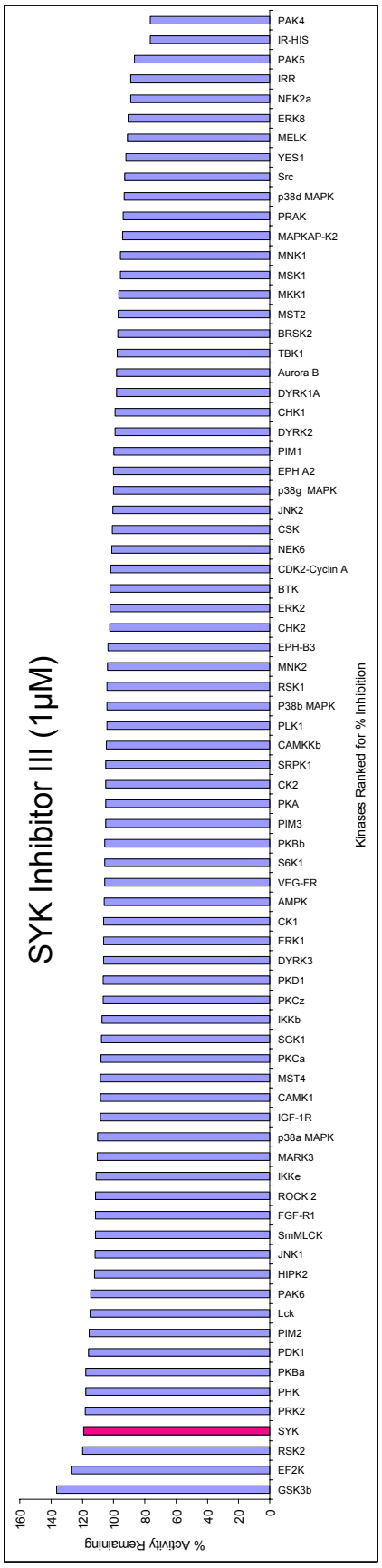
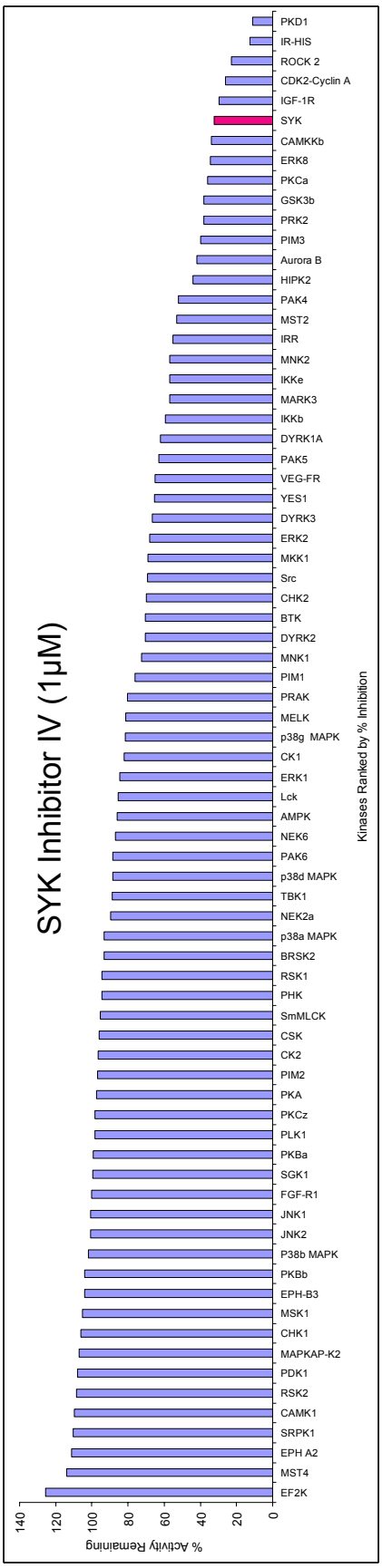
SYK inhibitor I  Chemical formula $C_{18}H_{15}N_3O_3S$ SYK IC_{50} = 14nM Original Ref: Lai JY (2003) Bioorg.Med.Chem Lett. 13:3111	SYK inhibitor II  Chemical formula $C_{14}H_{15}F_3N_6O•2HCl•2H_2O$ SYK IC_{50} = 41nM Original Ref: Hisamichi H (2005) Bioorg. Med. Chem 13:4936
SYK inhibitor III  Chemical formula $C_9H_7NO_4$ SYK IC_{50} = 2.5μM Original Ref: Wang WY (2006) Mol. Pharmacol. 70:1380	SYK inhibitor IV  Chemical formula $C_{20}H_{18}N_6O_3•HCl•H_2O$ SYK IC_{50} = 10nM Original Ref: Yamamoto N (2003) J Pharmacol Exp Ther 306:1174
R406  Chemical formula $C_{22}H_{23}FN_6O_5•C_6H_6O_3S$ SYK IC_{50} of 41nM. Original Ref: Braselmann S (2006) J Pharmacol Exp Ther 319:998	Piceatannol  Chemical formula $C_{14}H_{12}O_4$ SYK IC_{50} = 3μM Original Ref: Oliver JM *et al* (1994) J Bio Chem 269:29697

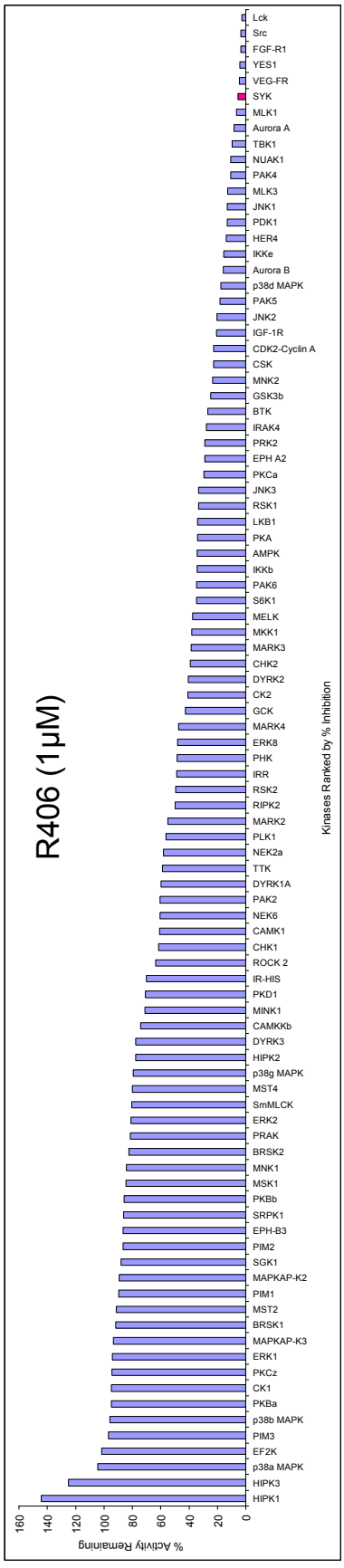
Figure 8 Kinase Specificity Graphs for SYK Inhibitors I, II, III, IV, R406 and Piceatannol.

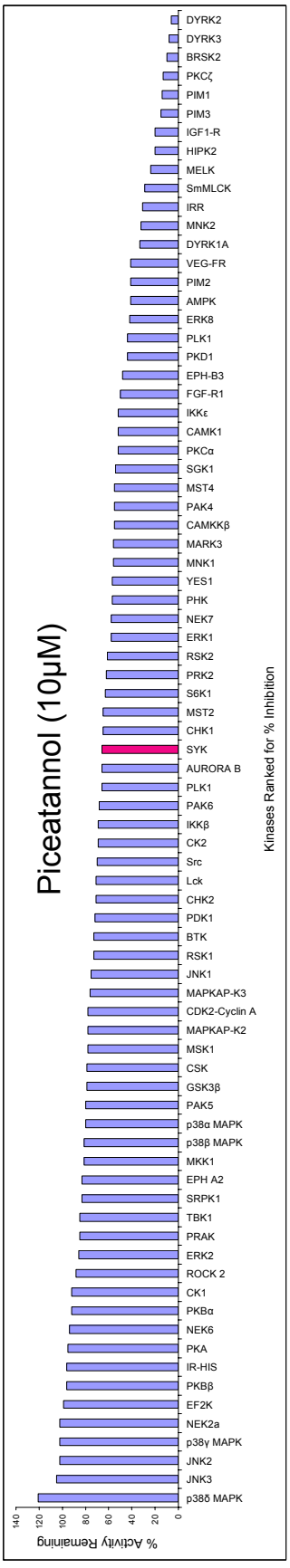





SYK Inhibitor III (1µM)
Kinases Ranked for % Inhibition
% Activity Remaining
160
140
120
100
80
60
40
20
0
PAK4
IR-HIS
PAK5
IRR
NEK2a
ERK8
MELK
YES1
Src
p38d MAPK
PRAK
MAPKAP-K2
MNK1
MSK1
MKK1
MST2
BRSK2
TBK1
Aurora B
DYRK1A
CHK1
DYRK2
PIM1
EPH A2
p38g MAPK
JNK2
CSK
NEK6
CDK2-Cyclin A
BTK
ERK2
CHK2
EPH-B3
MNK2
RSK1
P38b MAPK
PLK1
CAMKKb
SRPK1
CK2
PKA
PIM3
PKBb
S6K1
VEG-FR
AMPK
CK1
ERK1
DYRK3
PKD1
PKCz
IKKb
SGK1
PKCa
MST4
CAMK1
IGF-1R
p38a MAPK
MARK3
IKKe
ROCK 2
FGF-R1
SmMLCK
JNK1
HIPK2
PAK6
Lck
PIM2
PDK1
PKBa
PHK
PRK2
SYK
RSK2
EF2K
GSK3b


SYK Inhibitor IV (1µM)
Kinases Ranked by % Inhibition
% Activity Remaining
140
120
100
80
60
40
20
0
PKD1
IR-HIS
ROCK 2
CDK2-Cyclin A
IGF-1R
SYK
CAMKKb
ERK8
PKCa
GSK3b
PRK2
PIM3
Aurora B
HIPK2
PAK4
MST2
IRR
MNK2
IKKe
MARK3
IKKb
DYRK1A
PAK5
VEG-FR
YES1
DYRK3
ERK2
MKK1
Src
CHK2
BTK
DYRK2
MNK1
PIM1
PRAK
MELK
p38g MAPK
CK1
ERK1
Lck
AMPK
NEK6
PAK6
p38d MAPK
TBK1
NEK2a
p38a MAPK
BRSK2
RSK1
PHK
SmMLCK
CSK
CK2
PIM2
PKA
PKCz
PLK1
PKBa
SGK1
FGF-R1
JNK1
JNK2
P38b MAPK
PKBb
EPH-B3
MSK1
CHK1
MAPKAP-K2
PDK1
RSK2
CAMK1
SRPK1
EPH A2
MST4
EF2K


R406 (1µM)
% Activity Remaining
Kinases Ranked by % Inhibition
Lck
Src
FGF-R1
YES1
VEG-FR
SYK
MLK1
Aurora A
TBK1
NUAK1
PAK4
MLK3
JNK1
PDK1
HER4
IKKe
Aurora B
p38d MAPK
PAK5
JNK2
IGF-1R
CDK2-Cyclin A
CSK
MNK2
GSK3b
BTK
IRAK4
PRK2
EPH A2
PKCa
JNK3
RSK1
LKB1
PKA
AMPK
IKKb
PAK6
S6K1
MELK
MKK1
MARK3
CHK2
DYRK2
CK2
GCK
MARK4
ERK8
PHK
IRR
RSK2
RIPK2
MARK2
PLK1
NEK2a
TTK
DYRK1A
PAK2
NEK6
CAMK1
CHK1
ROCK 2
IR-HIS
PKD1
MINK1
CAMKKb
DYRK3
HIPK2
p38g MAPK
MST4
SmMLCK
ERK2
PRAK
BRSK2
MNK1
MSK1
PKBb
SRPK1
EPH-B3
PIM2
SGK1
MAPKAP-K2
PIM1
MST2
BRSK1
MAPKAP-K3
ERK1
PKCz
CK1
PKBa
p38b MAPK
PIM3
EF2K
p38a MAPK
HIPK3
HIPK1
160
140
120
100
80
60
40
20
0

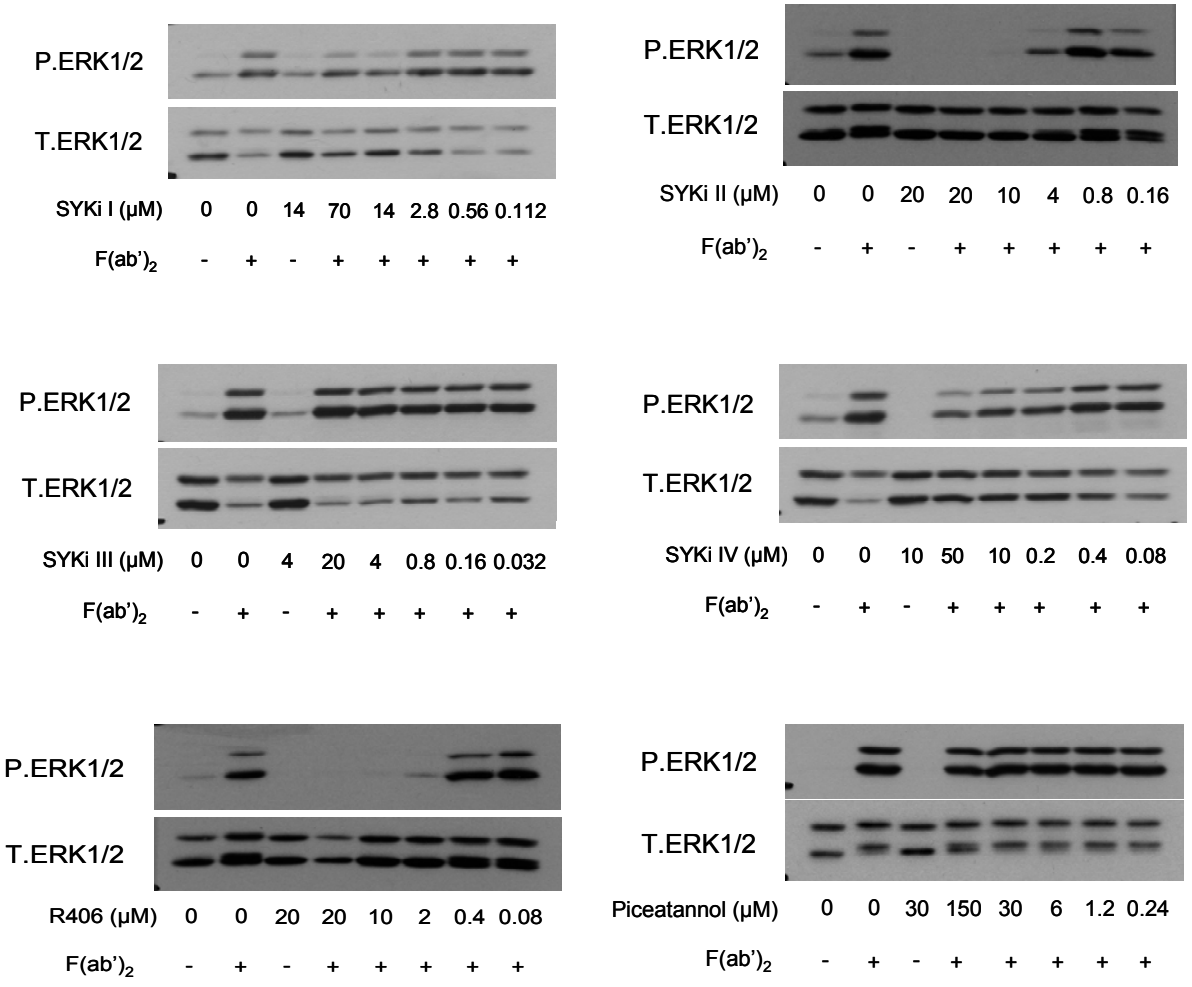

Piceatannol (10µM)
% Activity Remaining
Kinases Ranked for % Inhibition
DYRK2
DYRK3
BRSK2
PKCζ
PIM1
PIM3
IGF1-R
HIPK2
MELK
SmMLCK
IRR
MNK2
DYRK1A
VEG-FR
PIM2
AMPK
ERK8
PLK1
PKD1
EPH-B3
FGF-R1
IKKε
CAMK1
PKCα
SGK1
MST4
PAK4
CAMKKβ
MARK3
MNK1
YES1
PHK
NEK7
ERK1
RSK2
PRK2
S6K1
MST2
CHK1
SYK
AURORA B
PLK1
PAK6
IKKβ
CK2
Src
Lck
CHK2
PDK1
BTK
RSK1
JNK1
MAPKAP-K3
CDK2-Cyclin A
MAPKAP-K2
MSK1
CSK
GSK3β
PAK5
p38α MAPK
p38β MAPK
MKK1
EPH A2
SRPK1
TBK1
PRAK
ERK2
ROCK 2
CK1
PKBα
NEK6
PKA
IR-HIS
PKBβ
EF2K
NEK2a
p38γ MAPK
JNK2
JNK3
p38δ MAPK
140
120
100
80
60
40
20
0

4.3.1 SYK Inhibitors in A20 Cells

While *in vitro* screening can determine inhibitor selectivity, it is also necessary to titrate the inhibitor in cells. This is because differences in ATP concentrations between the cell and *in vitro* assays, as well as the ability of the inhibitor to enter the cell, affect the concentration required to inhibit SYK in cells. To investigate the effect of SYK inhibitors on the activation of SYK in cells, I decided to use a system in which SYK is fundamentally required – that is in the signalling downstream of activation of the B-cell receptor (Mocsai, Ruland et al. 2010). Using a murine B-cell lymphoma cell line (A20 cells) I stimulated the B-cell receptor with $F(ab')_2$ fragment rabbit anti-mouse IgG following pre-incubation with the relevant SYK inhibitor. Figure 9 shows that both R406 and SYK inhibitor II can inhibit ERK1/2 phosphorylation at concentrations above 2μM and 10μM respectively, and we can see that neither SYK inhibitor I, SYK inhibitor III nor piceatannol can prevent the activation of ERK1/2 at the concentrations used. SYK inhibitor IV does cause partial inhibition of ERK1/2 activation. These results are in keeping with the kinase specificity screening data, and for my ongoing experiments I decided to use only SYK inhibitor II and R406 as these both showed the best SYK inhibition in this B cell system.

Figure 9 SYK Inhibitors in A20 Cells

The cells were pre-incubated for 1 hour with the stated concentrations of SYK inhibitor I, SYK inhibitor II, SYK inhibitor III, SYK inhibitor IV, R406 or piceatannol. The B-cell receptor was then stimulated for 30min with $F(ab')_2$ fragment rabbit anti-mouse IgG. Cells were lysed in triton lysis buffer, protein concentration determined, and 20µg of protein denatured with SDS allowing the levels of the indicated proteins to be determined by immunoblotting.



One very surprising result was that piceatannol, which is widely used in the literature as a SYK inhibitor, did not cause any SYK inhibition in the B cell system. This was unexpected, and so to ensure there were no confounding factors within the source of the inhibitor I confirmed my findings by using piceatannol from both Calbiochem and Sigma (data not shown). Both sources of piceatannol gave identical results. To try and find an explanation for this, I reviewed the literature that first described piceatannol as an inhibitor of SYK.

Piceatannol is a naturally occurring plant stilbene that was initially shown to inhibit purified thymocyte p40 kinase (the catalytic fragment of SYK) *in vitro* by competition for the tyrosine peptide/protein substrate binding site (Geahlen and McLaughlin 1989). Further *in vitro* assays showed that piceatannol was more selective for SYK than the Src family kinase Lyn, as measured by autophosphorylation in anti-kinase immune complexes and by phosphorylation of a common peptide substrate (angiotensin I) (Oliver, Burg et al. 1994). Following this, the effects of piceatannol were studied in RBL-2H3 cells. Here the authors showed that piceatannol caused a dose dependent inhibition of the *in vivo* phosphorylation of SYK and most other cellular proteins (the doses of piceatannol used were from10µg/ml to 50µg/ml). Concentrations of piceatannol that inhibited the antigen-stimulated phosphorylation of SYK and multiple other substrates caused a general inhibition of receptor mediated metabolic and functional responses in mast cells (Oliver, Burg et al. 1994). SYK is known to be activated by collagen stimulation in platelets, and Keely *et al* used piceatannol to clarify the substrates of SYK in this setting. Using immune complex kinase assays they showed that pretreatment of platelets with

piceatannol at very high concentrations (50μg/ml) inhibited SYK kinase activity, measured by autophosphorylation and by the phosphorylation of the exogenous substrate tubulin, while not affecting Src kinase activity. However, using piceatannol at lower concentrations (10μg/ml) did not significantly inhibit SYK kinase activity, but did inhibit the tyrosine phosphorylation of SYK (Keely and Parisle 1996), suggesting that piceatannol can also affect other kinases in platelets too. More recently, the antioxidant effect of piceatannol has been investigated. Initially, the mechanism by which piceatannol is able to scavenge free radicals was investigated using Density Functional Theory (in depth account in (Piotrowska, Kucinska et al. 2012). Following this, the antioxidant effect of piceatannol in cells was tested, with pretreatment of piceatannol able to significantly inhibit the incidence of DNA single strand breaks induced by H_2O_2 in three separate leukemic cell lines (Ovesna, Kozics et al. 2006). This antioxidant effect was also present in melanoma B16 cells, which showed significant decreases in reactive species generation in cell culture supernatant following piceatannol treatment (Yokozawa and Kim 2007). Further, it was shown that piceatannol had a potent anti-melanogenic effect, as it was able to decrease melanin content without affecting cell viability (Yokozawa and Kim 2007), allowing suggestion that piceatannol may be a potential treatment for hyperpigmentation. From these studies, it is clear that piceatannol has a broad spectrum of biological activities, although its specificity for SYK has never been directly proven. It has been shown that the effect of piceatannol *in vitro* on SYK is greater than its effect on other Src family members, but that does not prove specificity or selectivity for SYK *in vivo*, and does not account for off target effects on other non-Src family kinases.

4.3.2 SYK Inhibitors in Wild Type BMDMs

Using the two most effective SYK inhibitors, SYK inhibitor II and R406, I next examined the effect of SYK inhibition on signalling pathways in wild type BMDMs following stimulation with zymosan, curdlan, depleted zymosan or LPS. As discussed earlier, Dectin-1 is reported to require SYK for the activation of downstream pathways. Thus, a SYK inhibitor should block signalling downstream of Dectin-1. The activation of the ERK1/2 and p38 pathways by depleted zymosan and curdlan are dependent upon Dectin-1 (figure 6), and consistent with an essential role for SYK downstream of Dectin-1, SYK inhibitor II blocked the activation of both ERK1/2 and p38 by either curdlan or depleted zymosan. In addition, SYK inhibitor II also blocked the phosphorylation of p105, a substrate of the IKK complex, and TBK1 (Figure 10).

LPS signals via TLR4 and would be expected to be able to activate signalling independently of SYK. Consistent with this, SYK inhibitor II did not affect LPS induced MAPK activation (figure 10). Zymosan signals via Dectin-1 and TLRs (figures 6 and 7) and as such, SYK inhibitor II had a slight effect on ERK1/2 and p38 activation by zymosan, whilst the phosphorylation of p105 was slightly reduced (figure 10). This is a similar pattern that we see in the Dectin-1 knockout in response to zymosan (figure 6).

The effect of R406 in wild type BMDMs is not as straightforward (figure 11). In response to LPS, R406 did not affect the activation of ERK1/2 or p38. In contrast it did reduce the phosphorylation of TBK1 on Ser172. Interestingly, TBK1 was one of the kinases inhibited by R406 in the *in vitro* screen.

In response to zymosan, R406 reduced the phosphorylation of ERK1/2, p38, p105 and TBK1. This would agree with the dual roles of TLR and Dectin-1 in the response to zymosan. The effects of R406 on curdlan or depleted zymosan stimulated macrophages however were surprising. Unlike SYK inhibitor II, R406 did not completely block curdlan or depleted zymosan pathway activation but instead changed its kinetics. The presence of R406 causes a significant time delay in activation of ERK1/2, p38, p105 and TBK1, i.e. R406 blocks initial phosphorylation of these proteins, but not the phosphorylation that occurs at later time points. Similar results were obtained in repeat experiments. There is no obvious explanation for this finding, although we must consider how R406 maybe metabolized or rendered inactive by the macrophages, or how any off target effects of the inhibitor may affect the activation of signalling cascades.

In both LPS and zymosan stimulated BMDMs R406 significantly decreased the activation of TBK1, which was not seen with SYK inhibitor II. These findings suggest that the inhibition of TBK1 is an off target effect of R406 rather than a consequence of SYK inhibition, but *in vivo* this off target effect on TBK1 may actually be contributing to the clinical efficacy seen with R406. The question therefore is does decreased Ser172 TBK1 phosphorylation have these profound effects on cytokine production? TBK1 is known, alongside IKKε, to be essential in regulating the production of type I interferons by phosphorylating the transcription factor IRF3 downstream of TLR3 and TLR4 activation (Hemmi, Takeuchi et al. 2004).

It has been suggested, based on over expression studies, that phosphorylation of TBK1 at Ser172 was an autophosphorylation event (Shimada, Kawai et al. 1999). More recently, through the use of a specific TBK1 inhibitor, BX795, it has been shown that Ser172 phosphorylation and activation of overexpressed TBK1 can be blocked by BX795 (Clark, Plater et al. 2009). However, BX795 can not suppress the Ser172 phosphorylation and activation of endogenous TBK1 in BMDMs in response to TLR3, TLR4, IL-1α or TNFα stimulation (Clark, Plater et al. 2009). This demonstrates that although TBK1 can autoactivate in an overexpression system, the activation of the endogenous TBK1 is by an upstream activating kinase that is as yet unidentified. It is therefore possible that R406 is targeting the activating upstream kinase of TBK1, whatever that maybe.

Figure 10 SYK Inhibitor II in Wild Type BMDMs

The cells were pre-incubated for 1 hour with 4μM SYK inhibitor II. The BMDMs were stimulated with 10μg/ml curdlan, 200μg/ml depleted zymosan, 200μg/ml zymosan and 100ng/ml LPS as indicated for the times stated. Cells were then lysed in 1% SDS-lysis buffer. The levels of the indicated proteins were determined by immunoblotting.

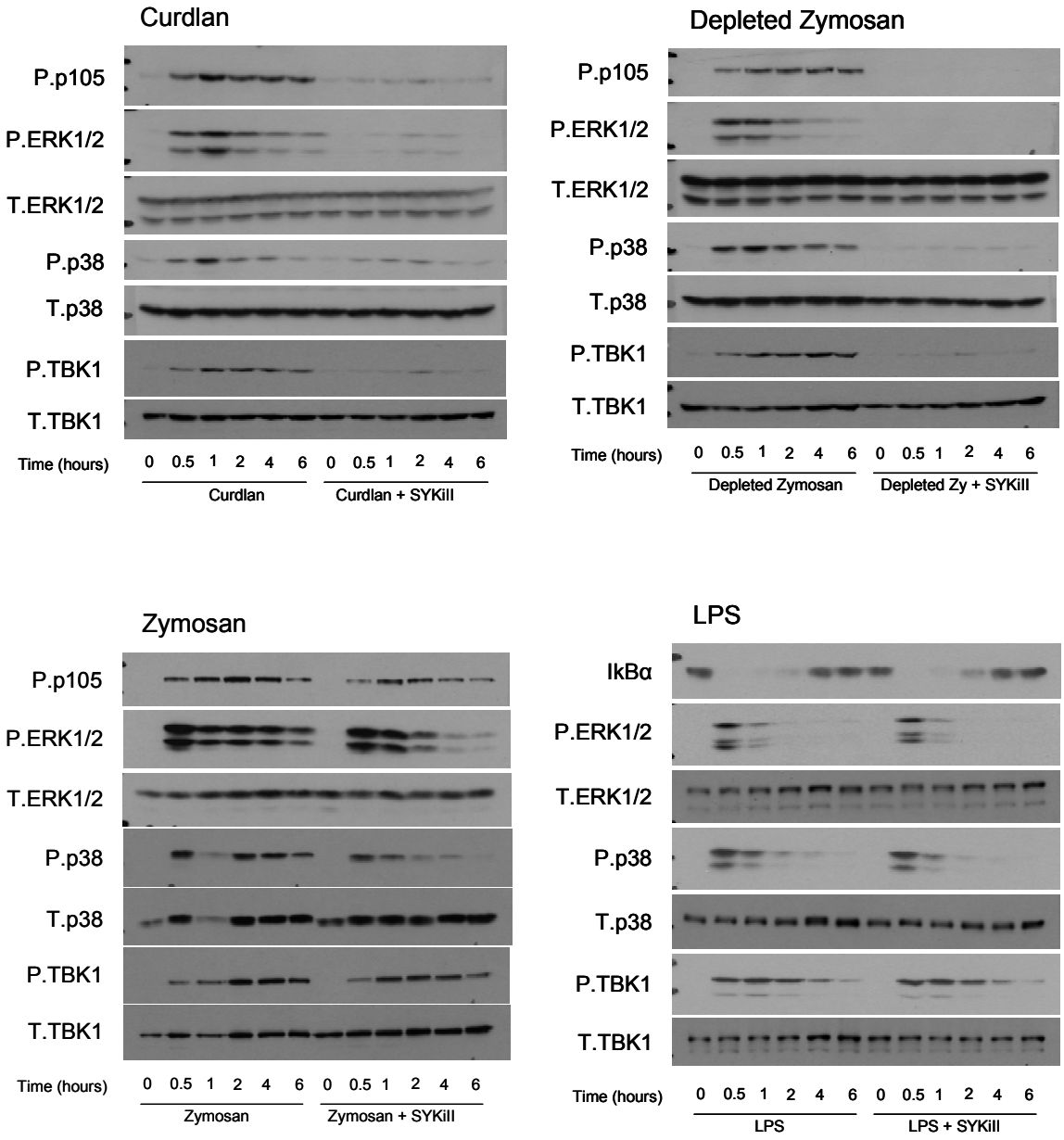
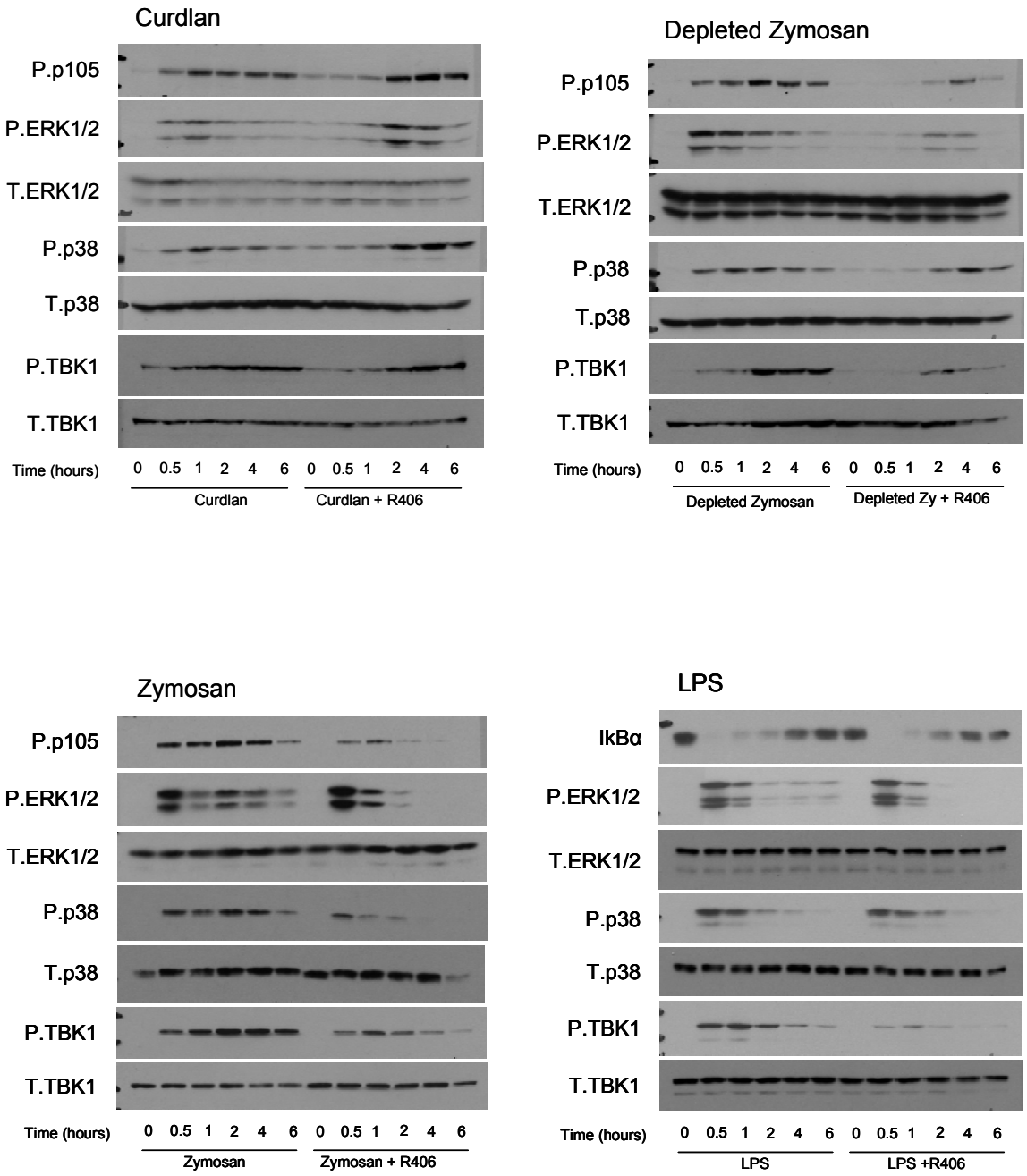


Figure 11 SYK Inhibitor R406 in Wild Type BMDMs

The cells were pre-incubated for 1 hour with 10μM R406. The BMDMs were stimulated with 10μg/ml curdlan, 200μg/ml depleted zymosan, 200μg/ml zymosan and 100ng/ml LPS as indicated for the times stated. Cells were then lysed in 1% SDS-lysis buffer. The levels of the indicated proteins were determined by immunoblotting.

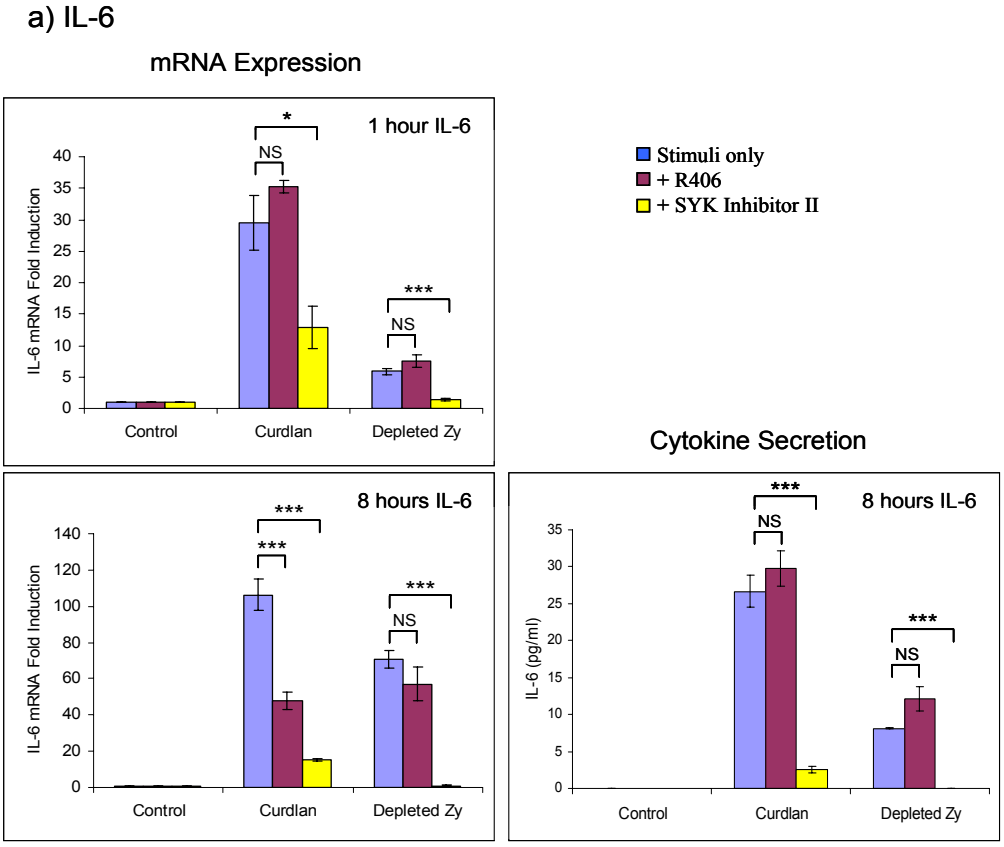


To asses the effect of SYK inhibitor II and R406 on cytokine expression and secretion I pre-incubated wild type BMDMs with either inhibitor and then stimulated with either curdlan, depleted zymosan, LPS, Pam3CSK4 or zymosan for 1 hour or 8 hours. As the levels of induction are very different between the Dectin-1 only agonists and the TLR agonists, I have analyzed the data separately. Looking at the Dectin-1 agonists curdlan or depleted zymosan stimulations (Figure 12), it is apparent that SYK inhibitor II is capable of significantly reducing the expression and secretion of IL-6, TNFα and IL-12 (p40), which is in keeping with the inhibition of MAPK and IKK activation seen by Western blotting in the presence of SYK inhibitor II (Figure 10). Interestingly, SYK inhibitor II also significantly decreases the expression and secretion of IL-10 (Figure 12). This suggests SYK inhibitor II causes a pan cytokine suppression, that is can suppress pro-inflammatory and anti-inflammatory cytokines equally, and as such, the effect *in vivo* of SYK inhibitor II cannot easily be predicted, and may not be beneficial for anti-inflammatory actions. The effect of R406 is not as clear, as its effect seems to be dependent upon the cytokine involved and the time point observed post stimulation (Figure 12). Like SYK inhibitor II, R406 blocked both TNFα mRNA induction and secretion in response to depleted zymosan or curdlan. R406 did not significantly affect IL-6 secretion, however it did result in a decrease in IL-6 mRNA levels at 8 hours following curdlan, but not depleted zymosan, stimulation. R406 did not block the initial induction of IL-10 mRNA at 1 hour but did greatly reduce IL-10 mRNA levels at 8 hours. Despite this, it did not affect IL-10 secretion in response to curdlan but did cause a small reduction in response to depleted zymosan.

In response to curdlan, the differences in IL-10 secretion at 8 hours versus mRNA expression at 1 and 8 hours may simply be a time delay, with the secretion shown at 8 hours actually reflecting the increase in expression seen earlier. Another possibility is that this increase in cytokine secretion is causing a feedback that may explain why R406 causes late activation of the MAPK signalling pathways (Figure 11). It would be interesting to repeat this experiment over a time course, because if this theory is correct, we would expect to see even bigger differences in expression at 4 hours.

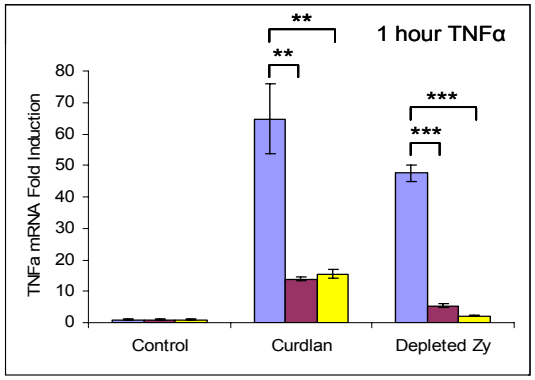
Figure 12 Cytokine mRNA Expression and Cytokine Secretion in Response to Curdlan or Depleted Zymosan in Wild Type BMDMs +/- SYK Inhibitors.

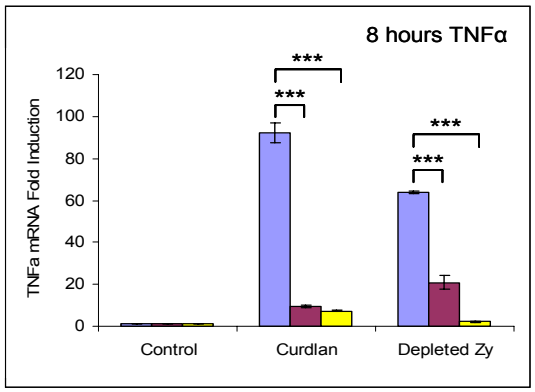
BMDMs incubated for 1 hour with inhibitors 4μM SYK inhibitor II or 10μM R406 prior to stimulation for 1 hour or 8 hours with either 10μg/ml curdlan or 200μg/ml depleted zymosan. Quantitative PCR of mRNA has the results normalized to expression of 18s RNA and the fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations (students t-test: NS is not significant; * p <0.05; ** p <0.01; *** p <0.001).



b) TNFα

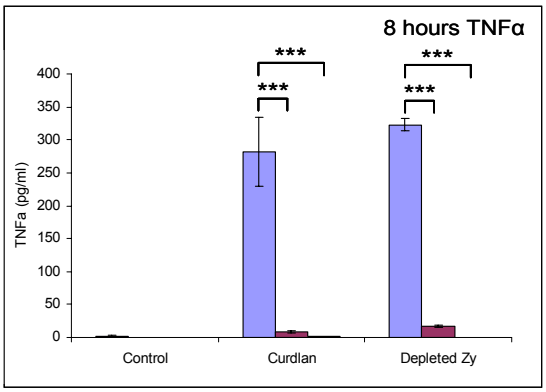
mRNA Expression





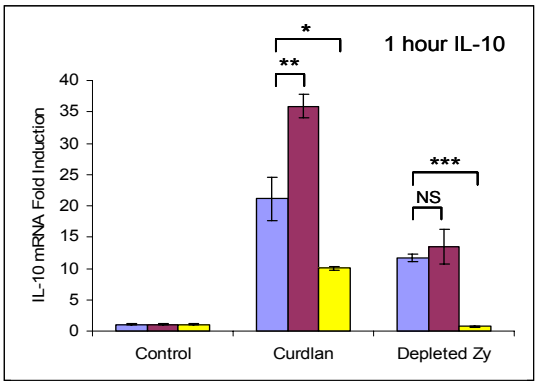
Stimuli only
+ R406
+ SYK Inhibitor II

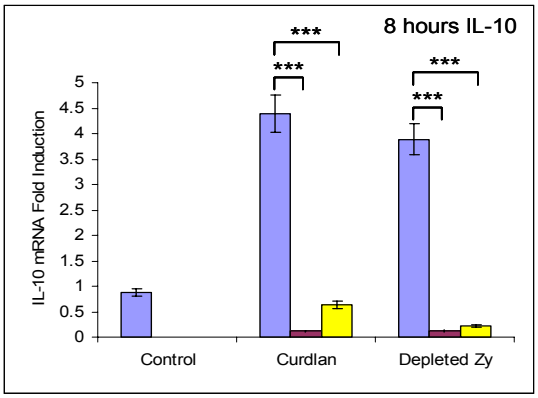
Cytokine Secretion



c) IL-10

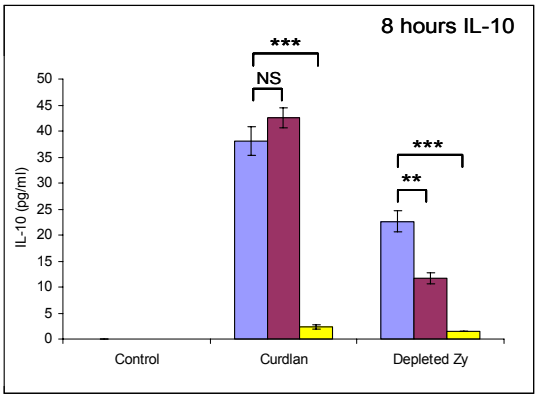
mRNA Expression



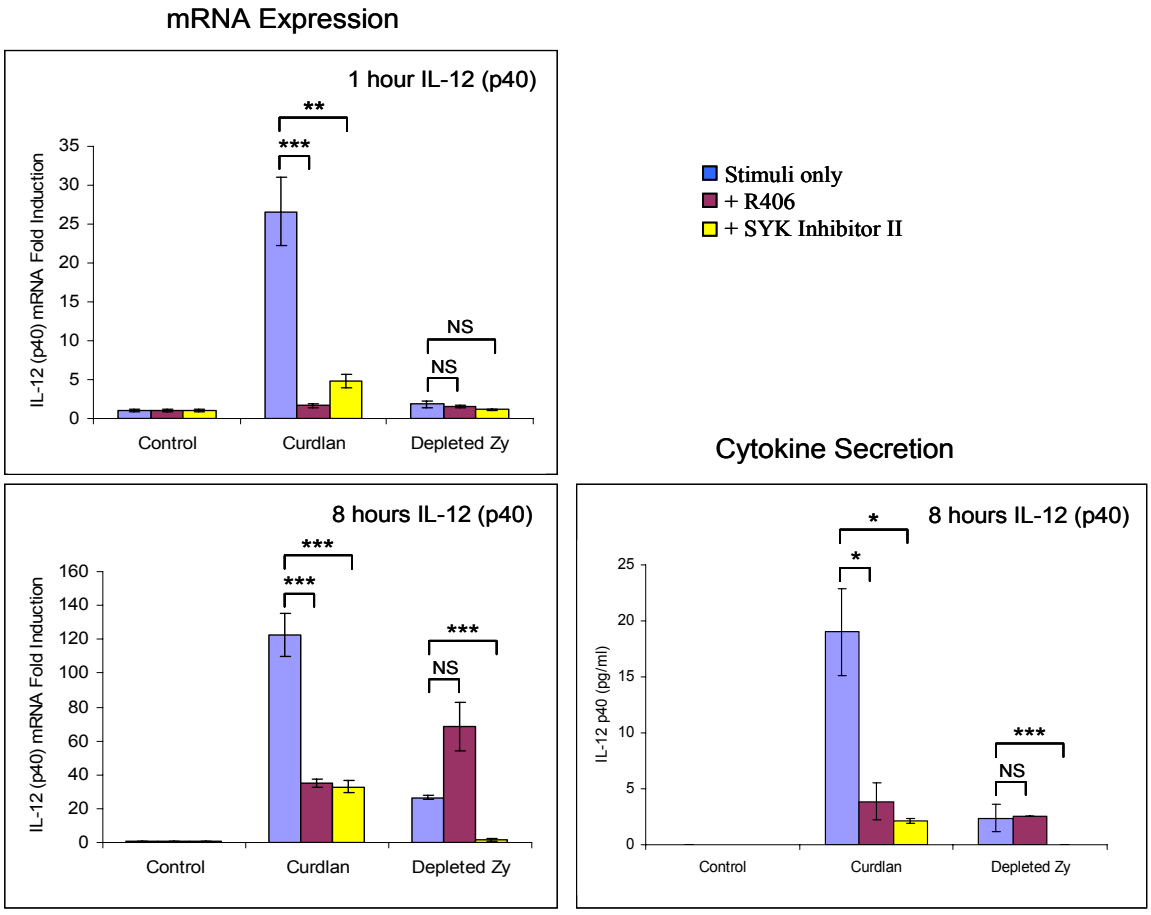


Stimuli only
+ R406
+ SYK Inhibitor II

Cytokine Secretion



d) IL-12 (p40)


mRNA Expression
1 hour IL-12 (p40)
IL-12 (p40) mRNA Fold Induction
35
30
25
20
15
10
5
0
**
***
NS
NS
Control
Curdlan
Depleted Zy
Stimuli only
+ R406
+ SYK Inhibitor II
8 hours IL-12 (p40)
IL-12 (p40) mRNA Fold Induction
160
140
120
100
80
60
40
20
0
***
***
***
NS
Control
Curdlan
Depleted Zy
Cytokine Secretion
8 hours IL-12 (p40)
IL-12 p40 (pg/ml)
25
20
15
10
5
0
*
*
***
NS
Control
Curdlan
Depleted Zy

Next, I investigated the effect of SYK inhibitor II and R406 in response to the TLR ligands Pam3CSK4 and LPS, and the TLR and Dectin-1 ligand zymosan (Figure 13). SYK inhibitor II is not effective at blocking TNFα or IL-6 expression in response to LPS or Pam3CSK4. SYK inhibitor II is more effective at blocking zymosan induced TNFα and IL-6 expression, but only at the 8 hour time point, which may be explained by SYK inhibitor II effect on the Dectin-1 component of zymosan signalling. Although SYK inhibitor II had only mild effects on IL-6 and TNF mRNA, it did strongly suppress their secretion in response to LPS, Pam3CSK4 and zymosan. This suggests that SYK inhibitor II targets either the translation or secretion of these cytokines rather than their transcription. SYK inhibitor II upregulated the expression of IL-10 at the 1 hour time point in response to LPS, Pam3CSK4 and zymosan, however, by 8 hours SYK inhibitor II repressed IL-10 mRNA and IL-10 secretion levels. SYK inhibitor II is able to significantly inhibit the expression and secretion of IL-12 (p40) at both 1 hour and 8 hours in response to LPS, Pam3CSK4 and zymosan, whilst its effect on other pro-inflammatory cytokines studied is limited. This suggests that perhaps IL-12 (p40) induction downstream of TLRs is linked to SYK (or another kinase targeted by SYK inhibitor II) that is not involved in the induction of the other pro-inflammatory cytokines I have measured.

R406 has a huge effect on the expression and secretion of pro-inflammatory cytokines in response to either LPS, Pam3CSK4 or zymosan (Figure 13), resulting in an almost complete suppression in expression and secretion of TNFα, IL-6 and IL-12 (p40). At 1 hour, R406 does cause a very modest increase in the expression of IL-10 in Pam3CSK4

or zymosan stimulated BMDMs, although by 8 hours R406 has significantly reduced (to control levels) the amount of IL-10 expressed, and the level of IL-10 secreted is minimal. It therefore seems very unlikely that the suppressive effect of R406 on pro-inflammatory cytokines is due to changes in IL-10. When analyzing the cytokine data along side the pathway activation Western blots (Figure 11) it becomes apparent that the effect of R406 is much greater than that expected by the modest effect on MAPK or NFκB signalling. Given that R406 has poor selectivity for SYK and yet has a much greater effect on cytokine production than SYK inhibitor II, it is likely that much of its effect is due to off target activities.

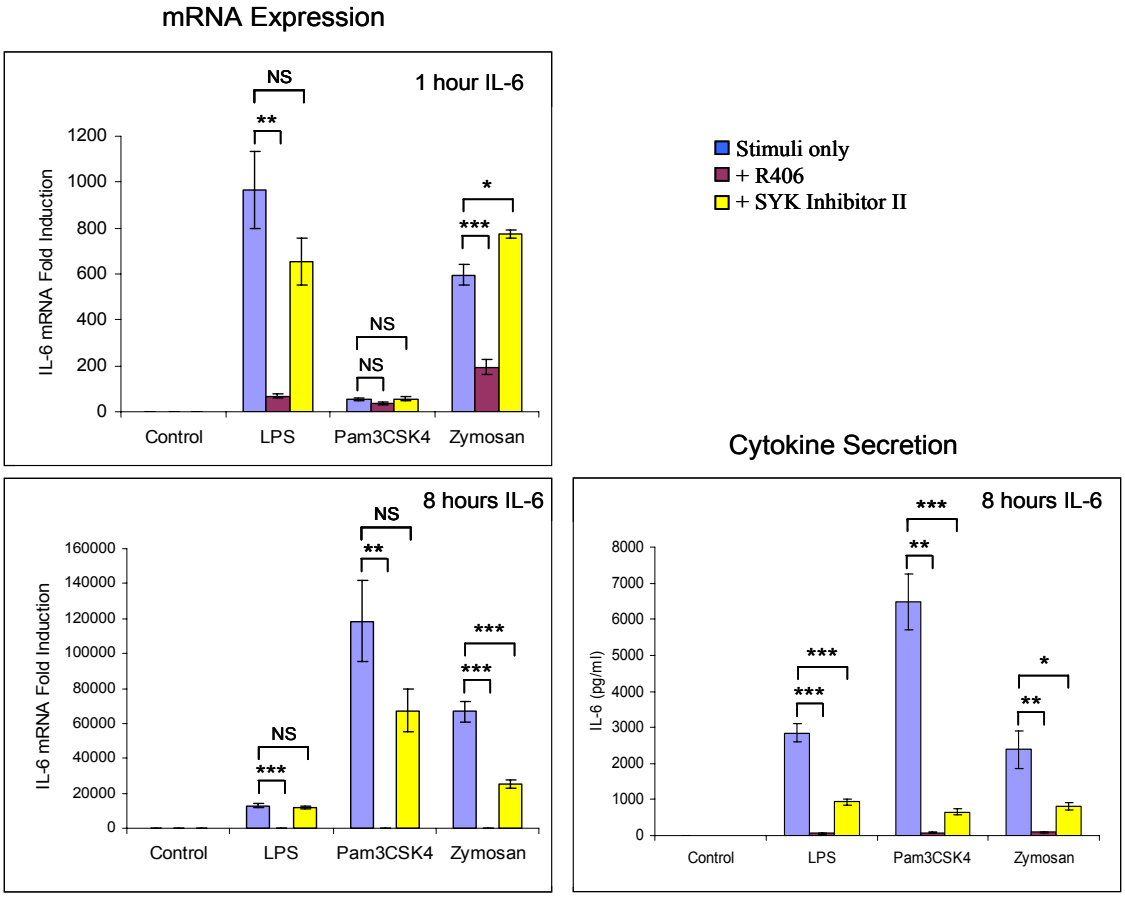
Further consideration needs to be given to the effect of both SYK inhibitors, but especially R406, on LPS stimulated BMDMs. As previously discussed, we see that in the presence of R406 p38, ERK1/2 and p105 are still fully activated whilst cytokine expression and secretion are suppressed. This scenario has been reported in the literature previously in the MyD88 or Trif knockout mice, where in response to LPS the MAPK pathways are still activated but the cytokine expression is significantly reduced. Looking at the kinase specificity screening data we could make suggestions of other kinases whose activities are also affected by R406, such as IKK. However, Figure 11 shows that IkBα degradation is unchanged in the presence of R406 which suggests that the IKK complex activity is unaffected by R406. We must remember that the kinase screening database has only looked at a fraction of the kinases at play, and there could be further targets of R406 that have not yet been discovered. Further, the effect of the inhibitor on a kinase *in vitro* may be different to the effect on that same kinase *in vivo*, as other effects such as cellular

ATP levels, concentrations of substrates and activators present will vary in the cellular system when compared to *in vitro* assays.

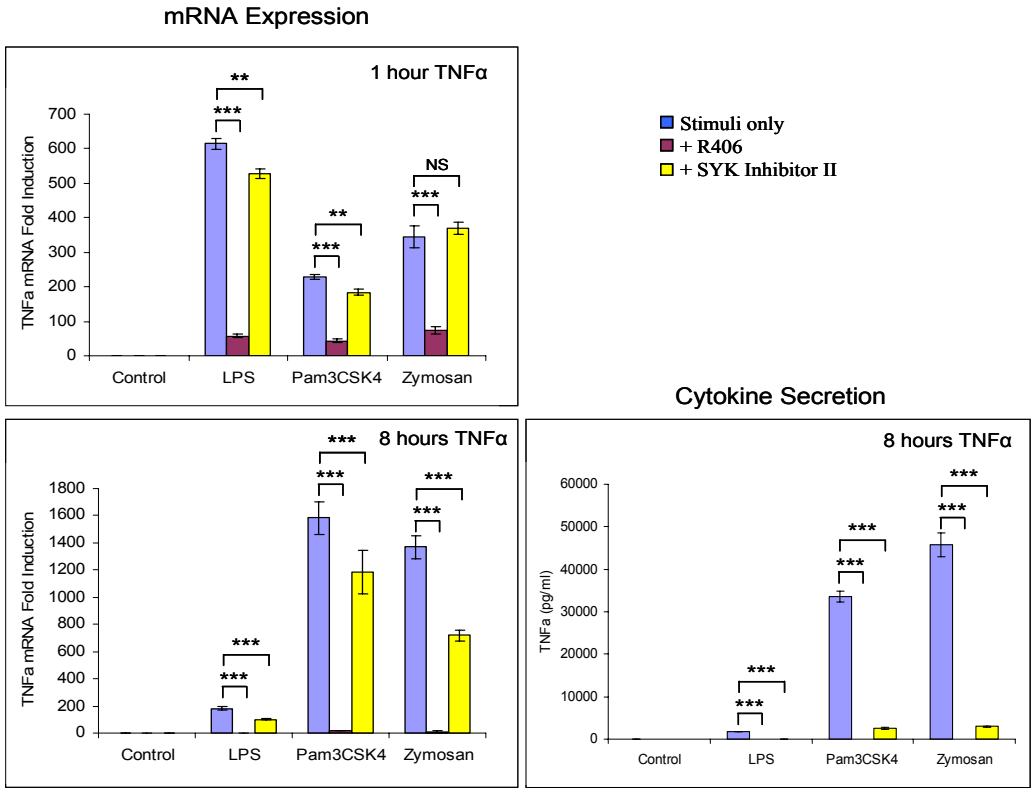
Figure 13 Cytokine mRNA Expression and Cytokine Secretion in Response to a Variety of Stimuli in Wild Type BMDMs +/- SYK Inhibitors.

BMDMs incubated for 1 hour with inhibitors 4µM SYK inhibitor II or 10µM R406 prior to stimulation for 1 hour or 8 hours with 100ng/ml LPS, 1µg/ml Pam3CSK4 or 200µg/ml zymosan. Quantitative PCR of mRNA has the results normalized to expression of 18s RNA and the fold induction calculated relative to unstimulated expression. Error bars show the SEM for 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

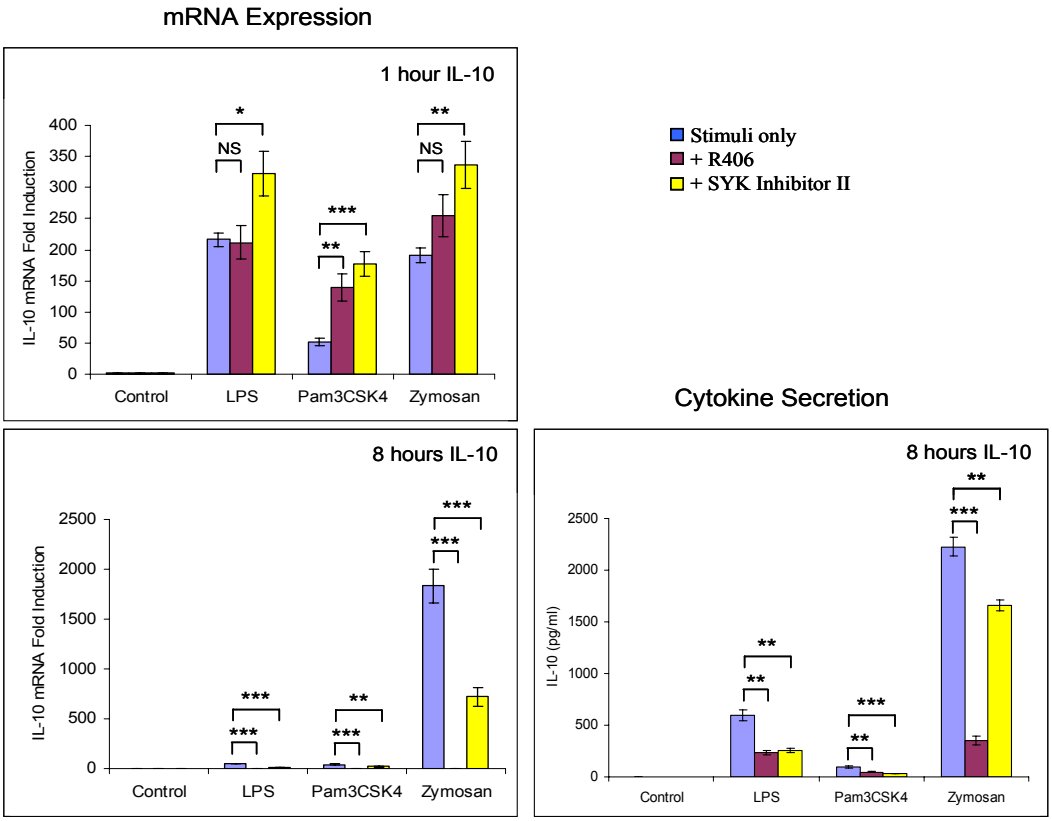
a) IL-6



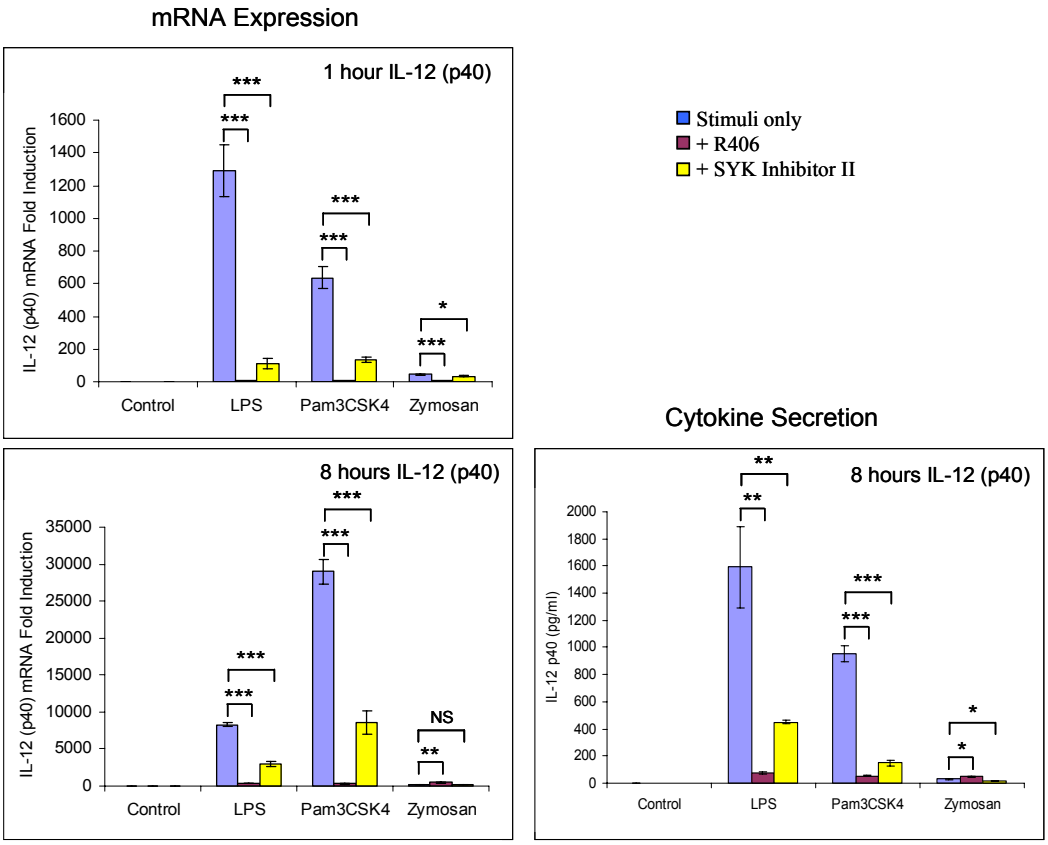
b) TNFα


mRNA Expression
1 hour TNFα
TNFa mRNA Fold Induction
Control
LPS
Pam3CSK4
Zymosan
Stimuli only
+ R406
+ SYK Inhibitor II
Cytokine Secretion
8 hours TNFα
TNFa mRNA Fold Induction
TNFa (pg/ml)

c) IL-10


mRNA Expression
1 hour IL-10
IL-10 mRNA Fold Induction
Control
LPS
Pam3CSK4
Zymosan
Stimuli only
+ R406
+ SYK Inhibitor II
Cytokine Secretion
8 hours IL-10
IL-10 (pg/ml)

d) IL-12 (p40)


mRNA Expression
1 hour IL-12 (p40)
IL-12 (p40) mRNA Fold Induction
1600
1400
1200
1000
800
600
400
200
0
Control
LPS
Pam3CSK4
Zymosan
***
*
Stimuli only
+ R406
+ SYK Inhibitor II
8 hours IL-12 (p40)
35000
30000
25000
20000
15000
10000
5000
NS
Cytokine Secretion
IL-12 p40 (pg/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
**

4.4 Dectin-1 Ligands Activate ERK1/2 via an Unidentified Kinase

For most stimuli, Raf-1 activates MEK1/2 which activates ERK1/2 (Kolch 2000). However, TLR signalling is unusual in that the pathway involves the serine/threonine kinase Tpl2. Before activation, Tpl2 is in an inactive complex with p105 and Abin2. TLR signalling activates IKKs which phosphorylates p105, causing it to dissociate from Tpl2 and allowing p105 proteolysis (Waterfield, Zhang et al. 2003). The activated Tpl2 activates MEK1/2 which in turn activates ERK1/2 (Das, Cho et al. 2005).It is not clear if signalling from Dectin-1 uses Raf-1 or Tpl2. It may be hypothesised that Raf-1 is responsible for activating ERK1/2, via MEK1/2, downstream of Dectin-1 as other ITAM containing receptors, for example the B-cell receptor, are known to require Raf-1 to activate ERK1/2 (Reviewed in (Kolch 2000). However, there is a report that shows that SYK is required for Tpl2 activation of ERK1/2 following TNFα stimulation of BMDMs (Eliopoulos, Das et al. 2005). As Dectin-1 utilises SYK, it is feasible that Tpl2 maybe the kinase responsible for the activation of ERK1/2 via MEK1/2. To investigate the potential roles of Raf-1 and Tpl2 downstream of Dectin-1 I initially used the Tpl2 inhibitor SHN681 (Hall, Kurdi et al. 2007).

Figure 14 shows that a concentration of 10μM of SHN681 is required to fully inhibit signalling from TLR4 (using LPS as an agonist). When zymosan was used as a stimulus 10μM of SHN681 was able to greatly reduce but not block ERK1/2 activation. Figure 15 shows that using the Dectin-1 only stimuli depleted zymosan 10μM of SHN681 can again

reduce, but not completely block ERK1/2 activation. This suggests that Dectin-1 may activate ERK1/2 through both Tpl2 dependent and independent mechanisms.

Figure 14 Zymosan and LPS Stimulation of Wild Type BMDMs with Tpl2 Inhibitor SHN681.

BMDMs were incubated with SHN681 at the stated concentrations for 1 hour prior to stimulation. The BMDMs were stimulated with either 200µg/ml zymosan or 100ng/ml LPS. BMDMs were lysed with 1% SDS lysis buffer and the levels of the indicated proteins were determined by immunoblotting.

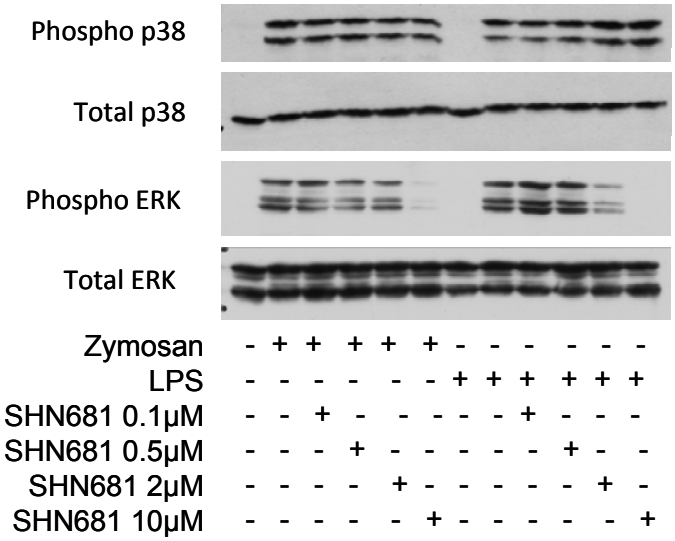
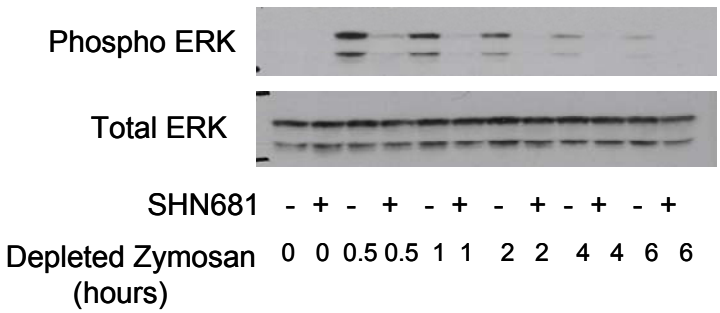


Figure 15 Depleted Zymosan in Wild Type BMDMs +/- the Tpl2 Inhibitor SHN681

BMDMs were incubated with 10µM SHN681 1 hour prior to stimulation with 200µg/ml depleted zymosan. BMDMs were lysed in 1% SDS lysis buffer and the levels of the indicated proteins were determined by immunoblotting.



To investigate the role of Tpl2 further, I used the small molecule IKKβ inhibitor BIX02514 to prevent the phosphorylation of p105 and block the release of active Tpl2 (Figure 16). With zymosan stimulation BIX02514 gives complete inhibition of IKKβ, as shown by the lack of phosphorylation of the IKKβ substrate p105, and the lack of degradation of IkappaBα. In the presence of BIX02514 zymosan does not activate ERK1/2 (Figure 16a). The Dectin-1 only stimuli curdlan (Figure 16b) and depleted zymosan (Figure 16c) show that BIX02514 can block the degradation of IkappaBα and phosphorylation of p105, whilst ERK1/2 remains fully activated. In contrast to the results with SHN681, this suggests that ERK1/2 is activated by a non-Tpl2 dependent mechanism downstream of Dectin-1.

To support the IKKβ inhibitor data, I utilised Abin2 knockout BMDMs. Abin2 forms a complex with p105 and Tpl2, and is required for activation of Tpl2. Figure 17a shows that in response to the TLR2 ligand Pam3CSK4 the Abin2 knockout BMDMs show greatly reduced ERK1/2 activation relative to the wild type BMDMs. Activation of p38 is however normal in the Abin2 knockout BMDMs. In response to zymosan, ERK1/2 activation is decreased when compared to the wild type, but there is still ERK1/2 phosphorylation (Figure 17b). The Dectin-1 only ligands curdlan (Figure 17c) and depleted zymosan (Figure 17d) show fractionally less ERK1/2 phosphorylation in the Abin2 knock out BMDMs, but this difference is very marginal. This offers further support to the hypothesis that there is an alternative pathway to Tpl2 responsible for the activation of ERK1/2 downstream of Dectin-1.

Figure 16 Wild Type BMDMs +/- IKKβ Inhibitor BIX02514

BMDMs were incubated with BIX02514 at a concentration of 10μM for 1 hour prior to stimulation with either a) 200μg/ml zymosan b) 10μg/ml curdlan or c) 200μg/ml depleted zymosan. BMDMs were then lysed in 1% SDS lysis buffer. The levels of the indicated proteins were determined by immunoblotting.

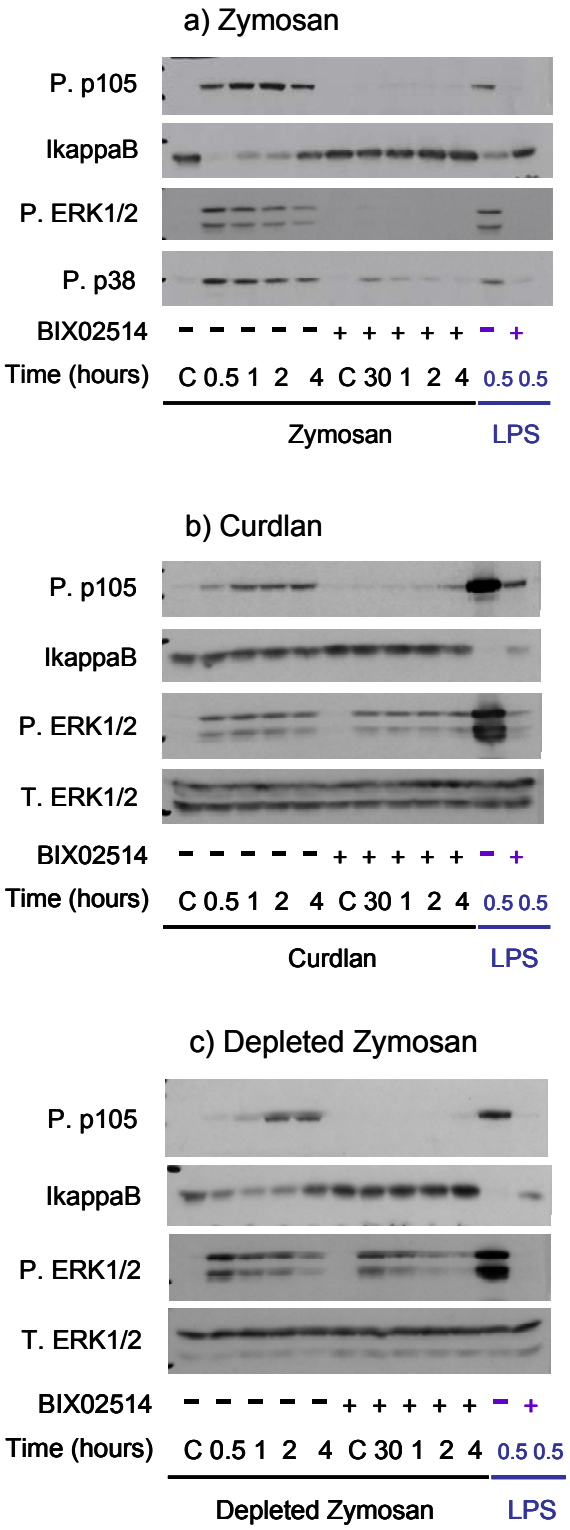
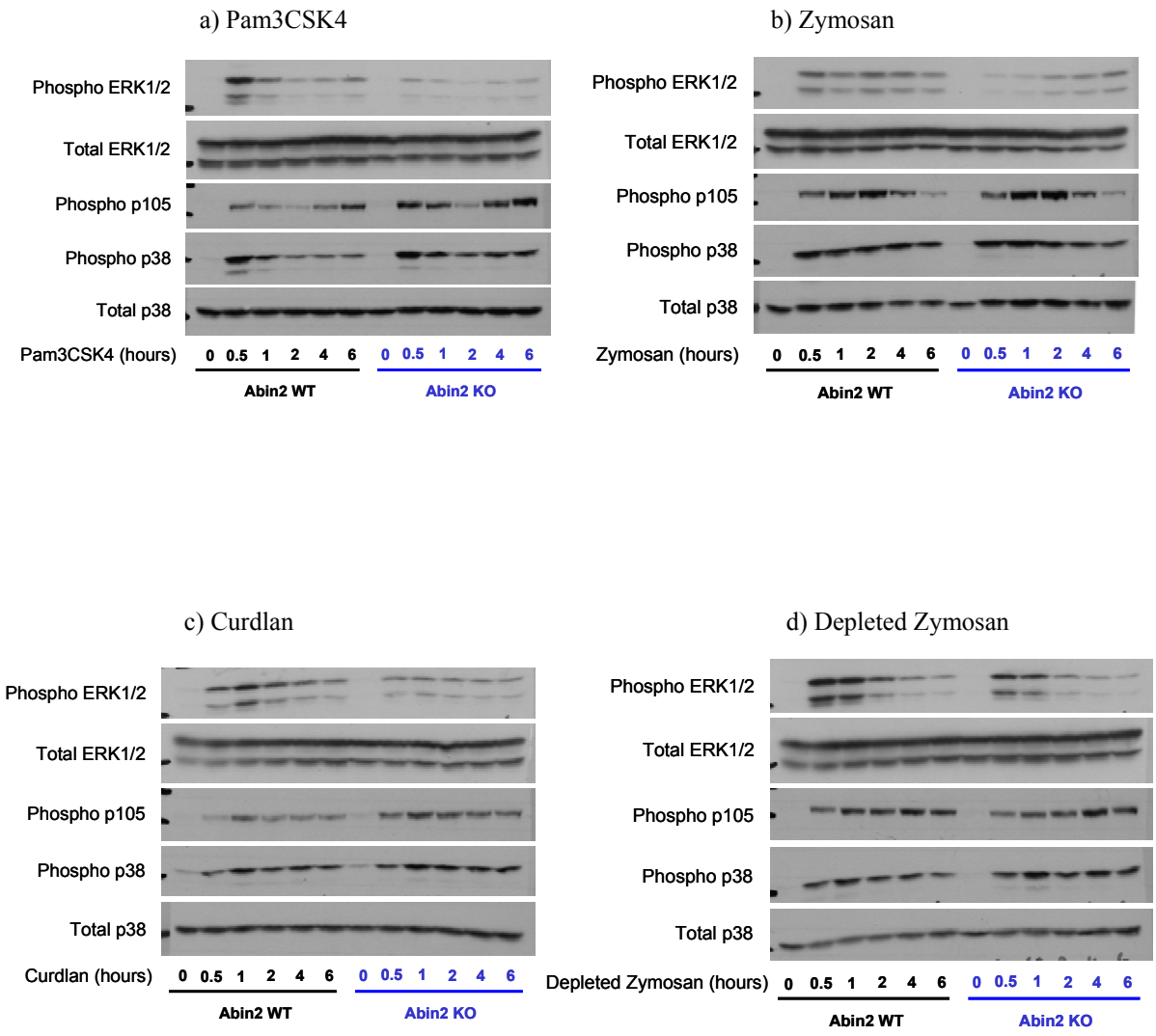


Figure 17 Abin2 Wild Type and Knockout BMDMs.

BMDMs were stimulated for the stated times with either a) 1µg/ml Pam3CSK4, b) 200µg/ml zymosan, c) 10µg/ml curdlan, or d) 200µg/ml depleted zymosan. BMDMs were then lysed in 1% SDS lysis buffer. The levels of the indicated proteins were determined by immunoblotting.



To clarify the role of Tpl2 in the activation of ERK1/2 downstream of Dectin-1 I isolated BMDMs from Tpl2 knock out mice. Figure 18a shows that as expected, in Pam3CSK4 stimulated Tpl2 knock out BMDMs p105 phosphorylation was apparent but no ERK1/2 activation was seen. In zymosan stimulated Tpl2 knock out BMDMs I saw no ERK1/2 activation which suggests that zymosan acts so strongly through TLR2 initially that we are unable to see the contribution of the Dectin-1 component of signalling (figure 18b). When the Tpl2 knock out BMDMs are stimulated only through Dectin-1 (figure 18 c&d), ERK1/2 activation is the same intensity as in the wild type BMDMs. This suggests that the activating pathway to ERK1/2 from Dectin-1 is not Tpl2 dependent. Interestingly, the Tpl2 inhibitor SHN681 is still able to inhibit the activation of ERK1/2 in the Tpl2 knock out BMDMs. It is therefore clear that the kinase responsible for the phosphorylation of ERK1/2 in this setting is an unidentified off target effect of SHN681.

The obvious hypothesis is that Raf-1 is the kinase responsible for the activation of MEK1/2 and then ERK1/2 downstream of Dectin-1. To investigate this further I stimulated wild type BMDMs with the ligand PMA, which is known to activate ERK1/2 via Raf-1. Figure 19 shows that although ERK1/2 phosphorylation is reduced by the addition of SHN681, it is not blocked. This shows that Raf-1 is inhibited by SHN681 to some extent, but not fully, and therefore there is likely to be another kinase involved. The involvement of Raf-1 however is notoriously difficult to prove, as most of the Raf-1 inhibitors available do not fully inhibit all isoforms of Raf-1 because, as it is so fundamental for cells, Raf-1 employs many feedback loops to both suppress and induce its own activation (Hall-Jackson, Goedert et al. 1999).

Figure 18 Tpl2 Wild Type and Knockout BMDMs.

BMDMs were pre-incubated with 10μM SHN681 1 hour prior to stimulation for the stated times with either a) 1μg/ml Pam3CSK4 b) 200μg/ml zymosan c) 10μg/ml curdlan d) 200μg/ml depleted zymosan. BMDMs were lysed with 1% SDS lysis buffer and levels of the indicated protein determined by immunoblotting.

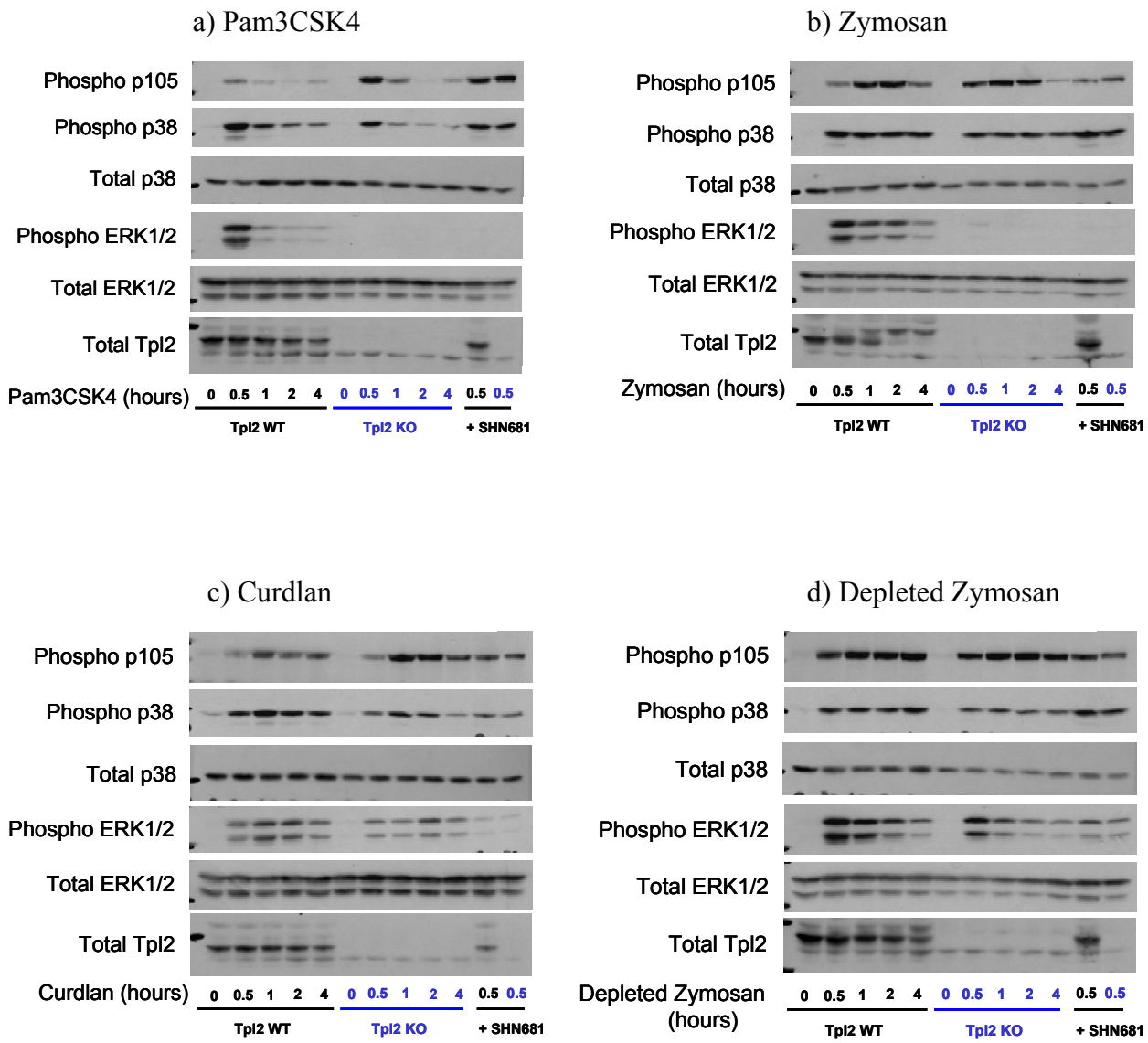
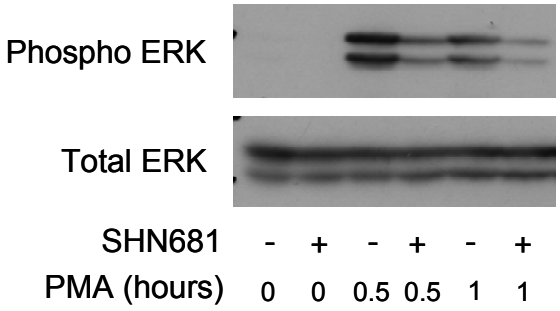


Figure 19 PMA Stimulation in Wild Type BMDMs +/- the Tpl2 Inhibitor SHN681

BMDMs were pre-incubated with 10μM SHN681 1 hour prior to stimulation for the stated times with 400ng/ml PMA. BMDMs were lysed with 1% SDS lysis buffer and levels of the indicated protein determined by immunoblotting.



4.5 The Role of MSK1/2

4.5.1 MSK1/2 is required to activate CREB in response to Dectin-1 ligands

Looking further downstream of the signalling pathway from Dectin-1 and TLR2, the role of MSK1/2 on the macrophages inflammatory response was investigated. MSK1/2 is activated by p38 and ERK1/2, and it has previously been shown that MSK1/2 is required for induction of IL-10, and hence suppression of pro-inflammatory cytokines, in response to LPS (Ananieva, Darragh et al. 2008). I was interested in investigating if MSK1/2 was also required for maximal IL-10 production in response to Dectin-1 stimulation.

To start, the small molecule p38 inhibitor SB203580 and the MEK inhibitor PD184352 were used to chemically inhibit MSK1/2 (Figure 20). In the wild type BMDMs, zymosan is able to activate MSK1, as judged by the phosphorylation of MSK1 on Thr581, a site that is critical for MSK1 activation. However, using the two inhibitors shows that both ERK1/2 and p38 are able to phosphorylate MSK1/2 in response to zymosan, and that this inhibition of MSK1/2 prevents the phosphorylation of CREB. This suggests the possibility that MSK1/2 are the only activators of CREB induced transcription following zymosan stimulation.

To confirm these findings, wild type and MSK1/2 knockout BMDMs were stimulated with zymosan over a 6 hour time course (Figure 21). As MSK1/2 is activated downstream of p38 and ERK1/2 we would not expect to see any difference in the activation of these proteins between the wild type and MSK1/2 knockout BMDMs. As predicted, the knockouts showed similar levels of ERK1/2, p38 and JNK activation to the wild type. However, while wild type BMDMs are able to phosphorylate CREB and the related transcription factor ATF1, this did not occur in the MSK1/2 knockout BMDMs. This confirms that MSK1/2 is the only activator of CREB induced transcription following zymosan stimulation.

Figure 20 Zymosan Stimulation in Wild Type BMDMs +/- MAPK Inhibitors

BMDMs were incubated for 1 hour with stated inhibitor prior to stimulation with 200µg/ml zymosan for either 1 hour or 8 hours. BMDMs were then lysed in 1% SDS lysis buffer and the levels of indicated proteins were determined by immunoblotting.

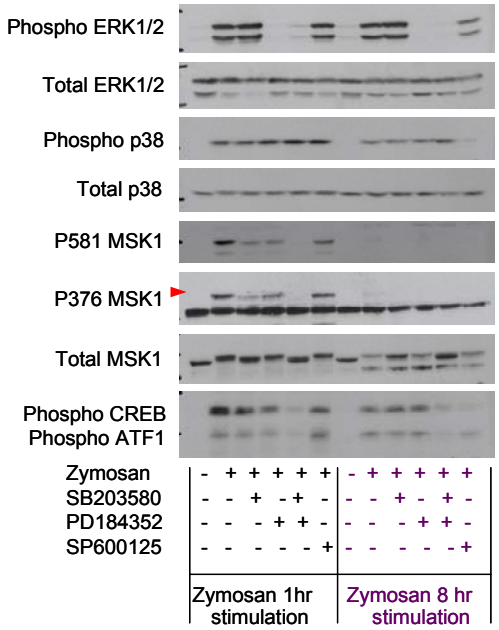
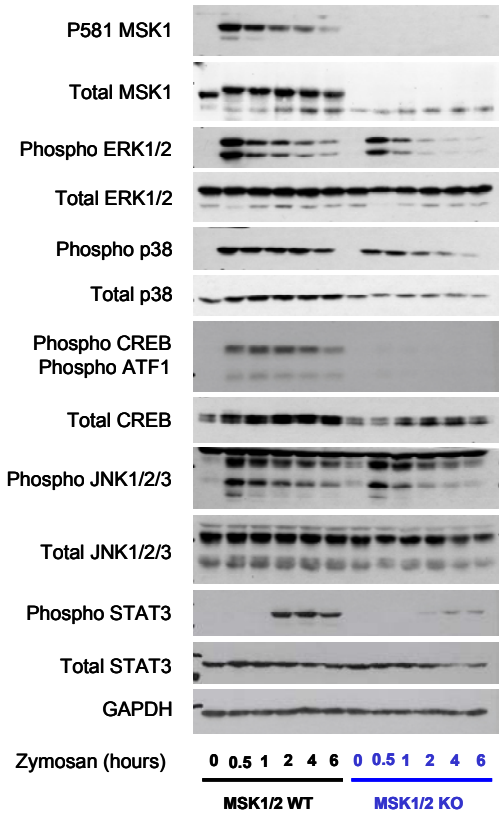


Figure 21 Zymosan Stimulation in Wild Type and MSK1/2 Knockout BMDMs

BMDMs were stimulated with 200µg/ml zymosan for the times stated. The BMDMs were lysed in 1% SDS lysis buffer and the levels of indicated proteins were determined by immunoblotting.



To determine if the effect of MSK1/2 activation on CREB phosphorylation seen with zymosan is only a result of TLR2 signalling, or if Dectin-1 was also involved, I firstly examined the effects of the small molecule p38 inhibitor SB203580 and the MEK inhibitor PD184352 on curdlan (Figure 22) and depleted zymosan (Figure 23) stimulated BMDMs. This showed that chemically inhibiting MSK1/2 with a combination of PD184352 and SB203580 was successful, as judged by lack of p581 MSK1 phosphorylation, and that this lack of MSK1 activation was associated with lack of CREB and ATF1 phosphorylation, notably at the 1 hour time point.

To confirm these results, I examined the effects of the Dectin-1 ligands curdlan or depleted zymosan on MSK1/2 knockout BMDMs. Figure 24 shows that in the MSK1/2 knockout BMDMs the response to curdlan resulted in both p38 and ERK1/2 being phosphorylated as the wild type, but that CREB was not phosphorylated at all. Depleted zymosan stimulation (Figure 25) supports this finding, showing that CREB is not activated in the MSK1/2 knock out BMDMs, whilst p38 and ERK1/2 are phosphorylated to the same level as the wild type BMDMs.

The conclusion from these experiments is that MSK1/2 is required to activate CREB in response to Dectin-1 stimulation.

Figure 22 Curdlan Stimulation in Wild Type BMDM +/- MAPK Inhibitors

BMDMs were incubated for 1 hour with stated inhibitor prior to stimulation with 10µg/ml curdlan for either 1 hour or 8 hours. BMDMs were then lysed in 1% SDS lysis buffer and levels of indicated proteins were determined by immunoblotting.

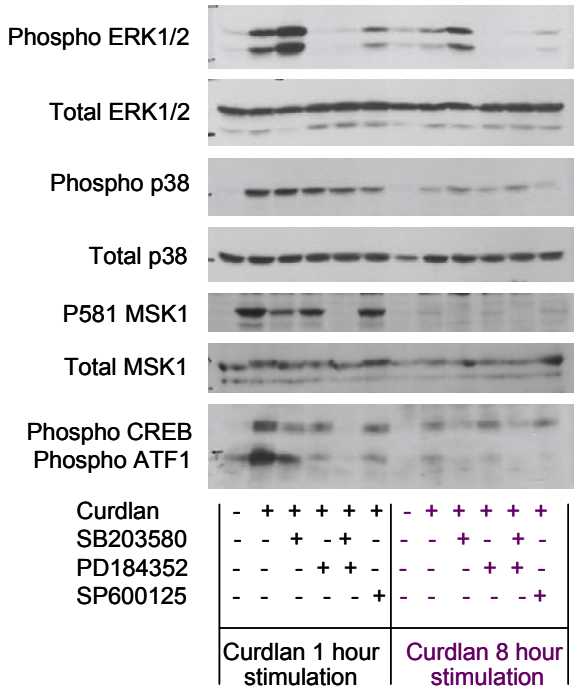


Figure 23 Depleted Zymosan Stimulation in Wild Type BMDMs +/- MAPK Inhibitors

BMDMs were incubated for 1 hour with stated inhibitor prior to stimulation with 200µg/ml depleted zymosan for either 1 hour or 8 hours. BMDMs were then lysed in 1% SDS lysis buffer and levels of indicated proteins were determined by immunoblotting

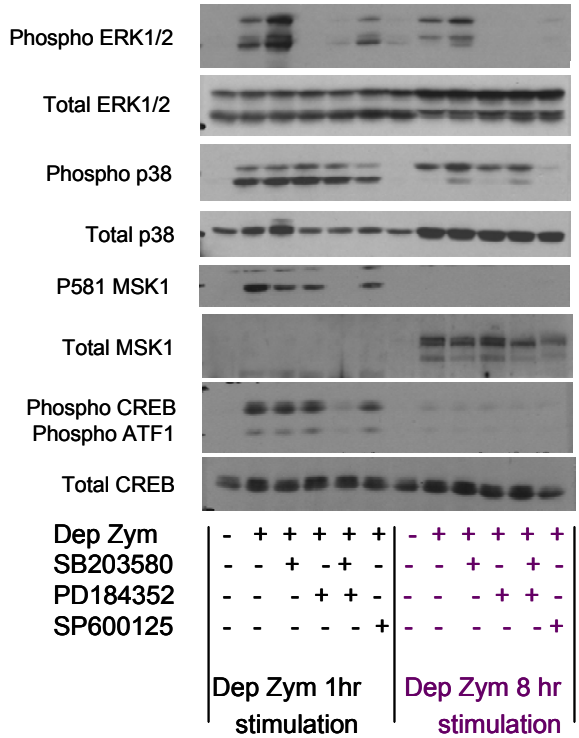


Figure 24 Curdlan Stimulation in Wild Type and MSK1/2 Knockout BMDMs
BMDMs were stimulated with 10µg/ml curdlan for the times stated. The BMDMs were lysed in 1% SDS lysis buffer and levels of indicated proteins were determined by immunoblotting

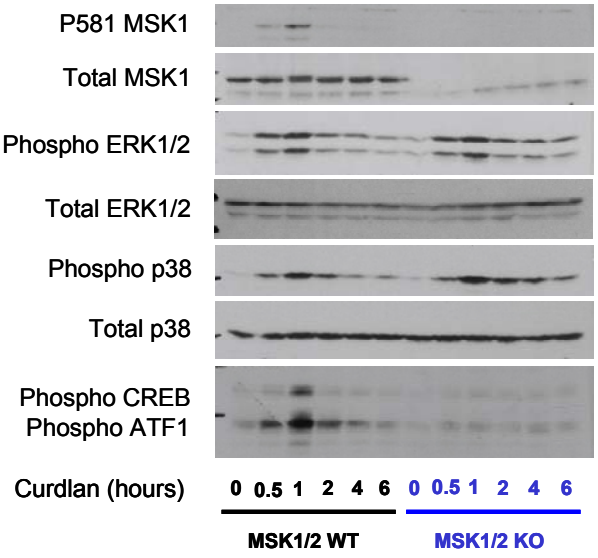
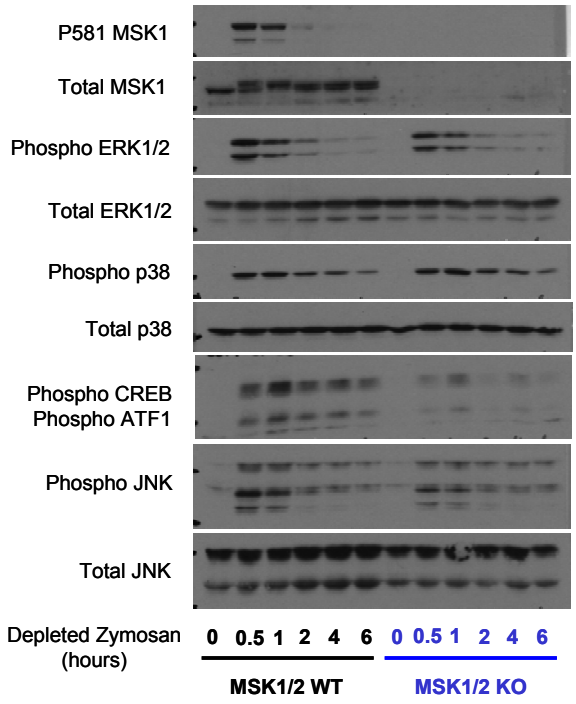


Figure 25 Depleted Zymosan Stimulation in Wild Type and MSK1/2 Knockout BMDMs
BMDMs were stimulated with 200µg/ml depleted zymosan for the times stated. The BMDMs were lysed in 1% SDS lysis buffer and levels of indicated proteins were determined by immunoblotting



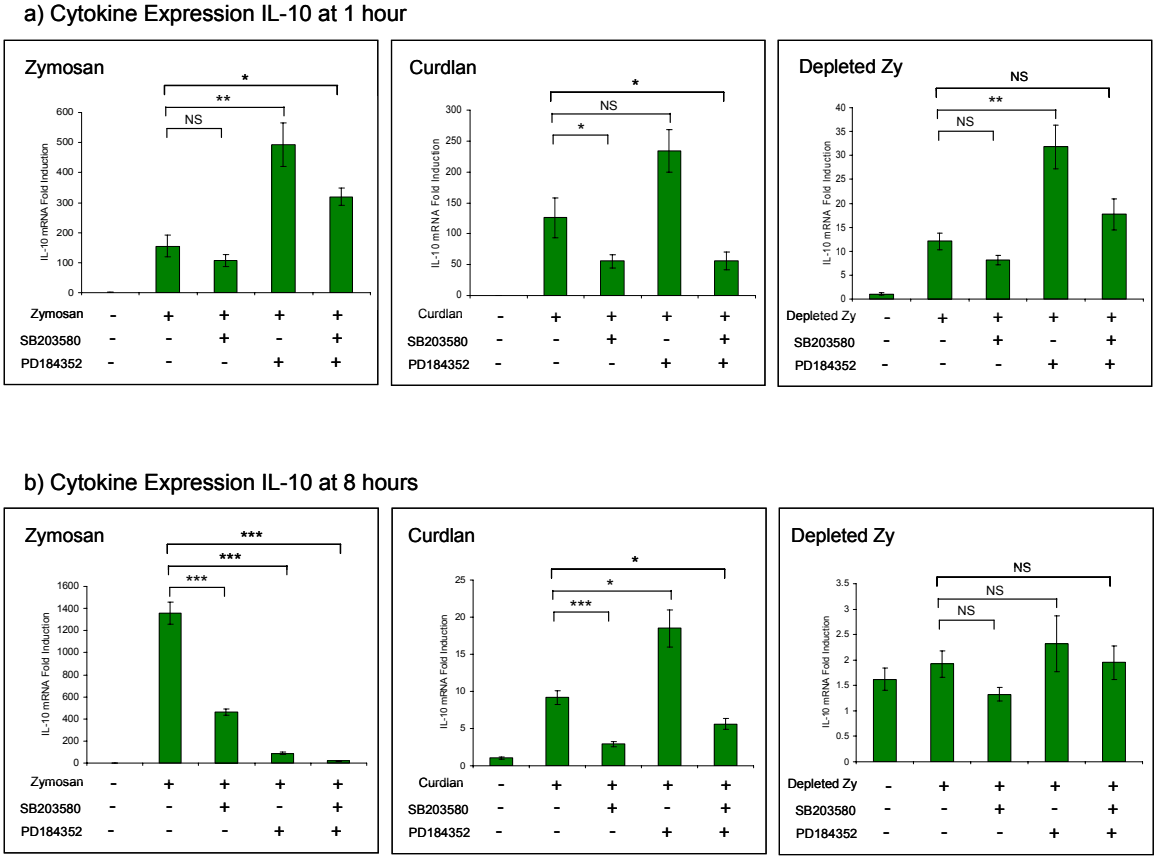
4.5.2 Effect of MSK1/2 on Cytokine Production

It has previously shown that in response to LPS, MSK1/2 knockout BMDMs show a significant increase in the pro-inflammatory cytokines TNFα, IL-6 and IL-12, and that this was directly a result of decreased expression and secretion of IL-10. It was also shown that in response to LPS, the lack of MSK1/2 prevents the activation of CREB and ATF1 which would, in normal circumstances, induce DUSP1, a MAPK phosphatase which inactivates p38 and JNK, and increase IL-10 expression (Ananieva, Darragh et al. 2008).

To assess the effect of the absence of MSK1/2 on the production of cytokines in response to Dectin-1 ligands, I firstly used a combination of the p38 inhibitor SB203580, and the MEK inhibitor PD184352 to chemically block MSK1/2. Figure 26 shows that following 1 hour of stimulation with either zymosan, curdlan or depleted zymosan the levels of IL-10 mRNA were upregulated. Pretreatment of BMDMs with PD184352 resulted in a significant increase in IL-10 mRNA levels in response to zymosan and depleted zymosan. SB203580 had little effect on its own, although a combination of SB203580 and PD184352 resulted in an inhibition of IL-10 mRNA levels relative to PD184352 alone. At 8 hours, in response to zymosan both SB203580 and PD184352 inhibit IL-10 expression and secretion, but the combination of the two inhibitors is able to suppress IL-10 expression and secretion to a much greater extent than either inhibitor individually. In response to the Dectin-1 ligand curdlan at 8 hours SB203580 was able to suppress IL-10 expression and secretion, however PD184352 actually caused greater IL-10 expression than the non-inhibitor treated BMDMs. At 8 hours, depleted zymosan showed the same

trends as curdlan, but the differences in expression levels were not significant. The unexpected effects of PD184352 were not due to a failure of the inhibitor, as the combination of SB203580 and PD184352 were able to successfully inhibit nur77, a known target gene of MSK1/2 (Figure 27).

Figure 26 a) and b) IL-10 mRNA Expression and c) IL-10 Secretion in Response to Dectin-1 Ligands in Wild Type BMDMs +/- MAPK Inhibitors. BMDMs incubated for 1 hour with inhibitors prior to stimulation for either 1 hour or 8 hours with either 200µg/ml zymosan, 10µg/ml curdlan or 200µg/ml depleted zymosan. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



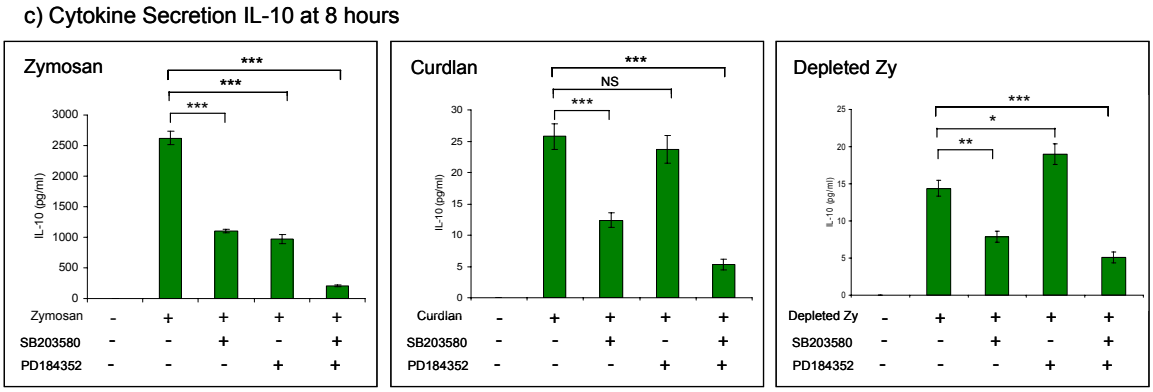
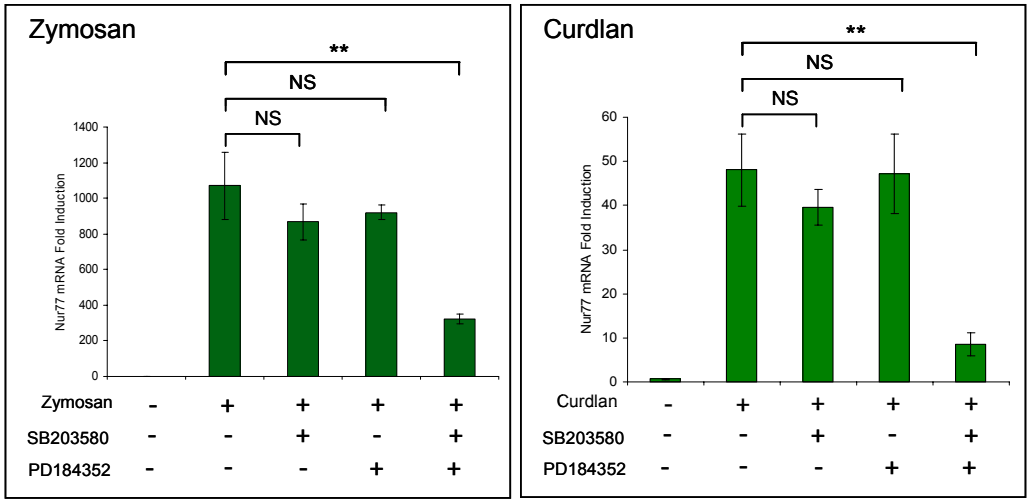
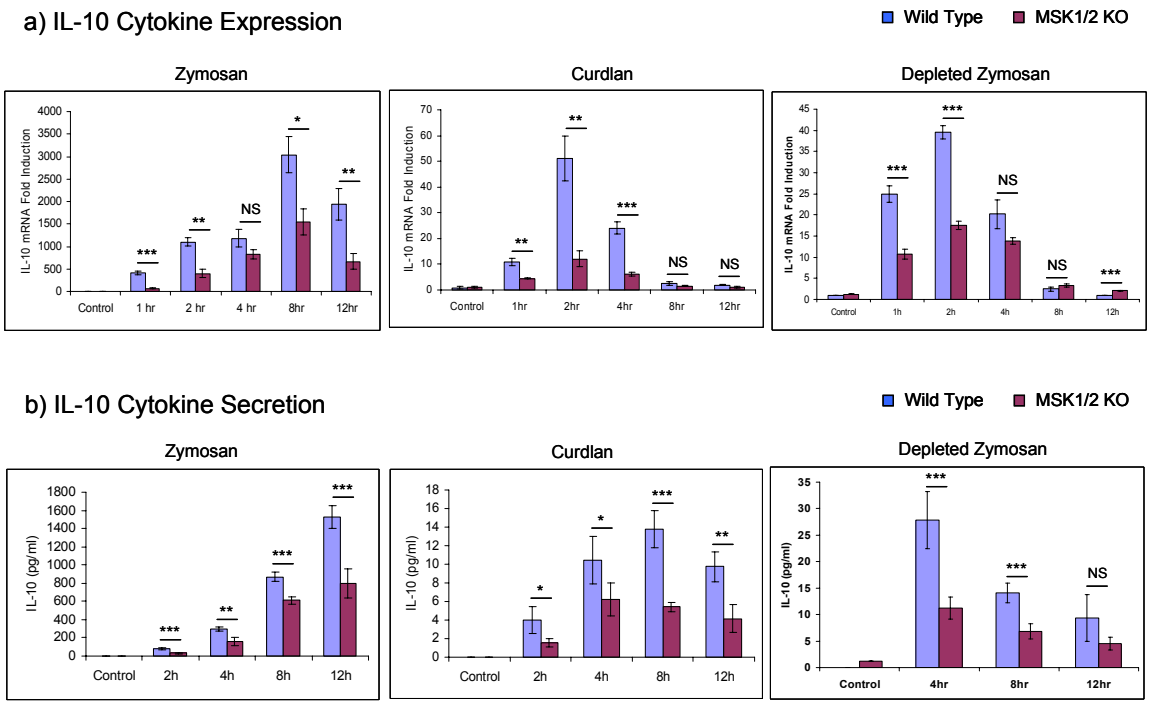


Figure 27 Nur77 mRNA Expression in Response to Dectin-1 Ligands in Wild Type BMDMs +/- MAPK Inhibitors. BMDMs incubated for 1 hour with inhibitors prior to stimulation for 1 hour with either 200µg/ml zymosan or 10µg/ml curdlan. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



The use of MAPK inhibitors to chemically prevent the activation of MSK1/2 shows that although the IL-10 expressed and secreted in response to Dectin-1 ligands is less in the dual inhibited BMDMS, it is not reduced to the same extent as previously reported with LPS stimuli (Ananieva, Darragh et al. 2008). To further investigate the requirement of MSK1/2, I examined the IL-10 response to Dectin-1 ligands in MSK1/2 knockout BMDMs (Figure 28). This shows that in response to zymosan, curdlan and depleted zymosan, the MSK1/2 deficient BMDMs both express less IL-10 mRNA and secrete significantly less IL-10 than the wild type BMDMs. Together, these results together would suggest that MSK1/2 activity is required for maximal IL-10 transcription, but that ERK1/2 is able to repress IL-10 transcription via a second MSK1/2 independent pathway.

Figure 28 a) IL-10 mRNA Expression and b) IL-10 Secretion in Response to Dectin-1 Ligands in MSK1/2 Wild Type and Knockout BMDMs. BMDMs stimulated for stated times with either 200µg/ml zymosan, 10µg/ml curdlan or 200µg/ml depleted zymosan. a) Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to wild type expression at 0h. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



IL-10 is known to be a potent anti-inflammatory cytokine, able to limit TNFα, IL-6 and IL-12 production following LPS stimulation of BMDMs (Ananieva, Darragh et al. 2008). It could therefore be expected that as Dectin-1 stimulated MSK1/2 deficient or inhibited BMDMs produce less IL-10, they would produce more pro-inflammatory cytokines. Figure 29 shows that zymosan stimulated BMDMs decrease the expression of IL-6 and TNFα when treated with the combination of SB203580 and PD184352. Curdlan and depleted zymosan show no difference in IL-6 mRNA expression in response to SB203580 alone, whilst with PD184352 treatment alone we see a rise in IL-6 transcription, but a non significant trend towards decreasing TNFα transcription. In response to curdlan or depleted zymosan, the combination of SB203580 and PD184352 together makes no significant difference to the level of IL-6 mRNA expression, however, in response to curdlan does produce a decrease in the levels of TNFα mRNA (Figure 29). The secretion profiles are more straight forward (Figure 30). In zymosan, curdlan or depleted zymosan stimulated BMDMs, pretreatment with either SB203580 or PD184352 alone causes a significant decrease in secreted TNFα and the combination of both SB203580 and PD184352 together reduces TNFα secretion to almost nothing. This is consistent with the known roles that p38 and ERK1/2 play in regulating TNFα translation and secretion (Kotlyarov, Neininger et al. 1999; Rousseau, Papoutsopopulou et al. 2008). In zymosan stimulated BMDMs, either SB203580 or PD184352 alone cause a decrease in the secreted levels of IL-6, but together they have a more profound effect. In curdlan stimulated BMDMs, neither SB203580 nor PD184352 alone had any effect at all, but in combination caused a significant reduction in the secreted levels of IL-6. The depleted zymosan treated BMDMs showed a slight decrease in IL-6 secretion in response to

SB203580 alone, but in combination with PD184352 had a more profound effect (Figure 30).

Figure 29 Cytokine mRNA Expression in Response to Dectin-1 Ligands in Wild Type BMDMs +/- MAPK Inhibitors. BMDMs incubated for 1 hour with inhibitors prior to stimulation for 8 hours with either 200µg/ml zymosan, 10µg/ml curdlan or 200µg/ml depleted zymosan. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

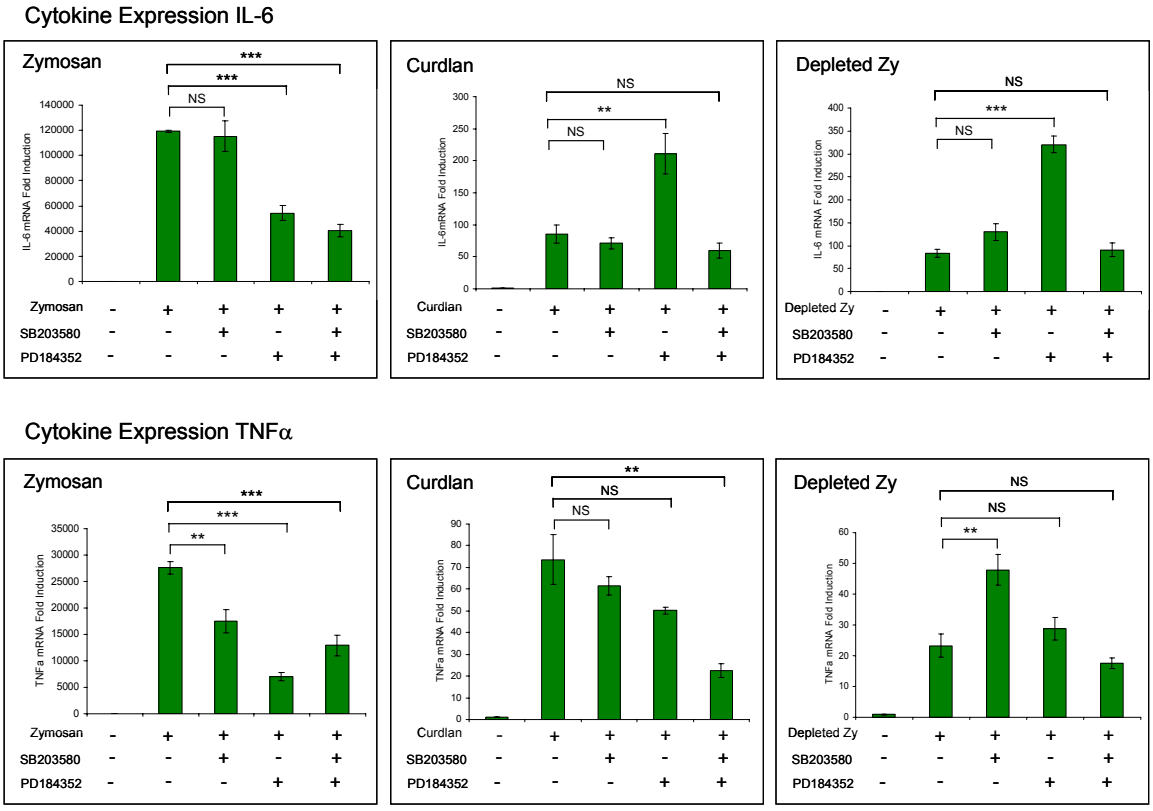
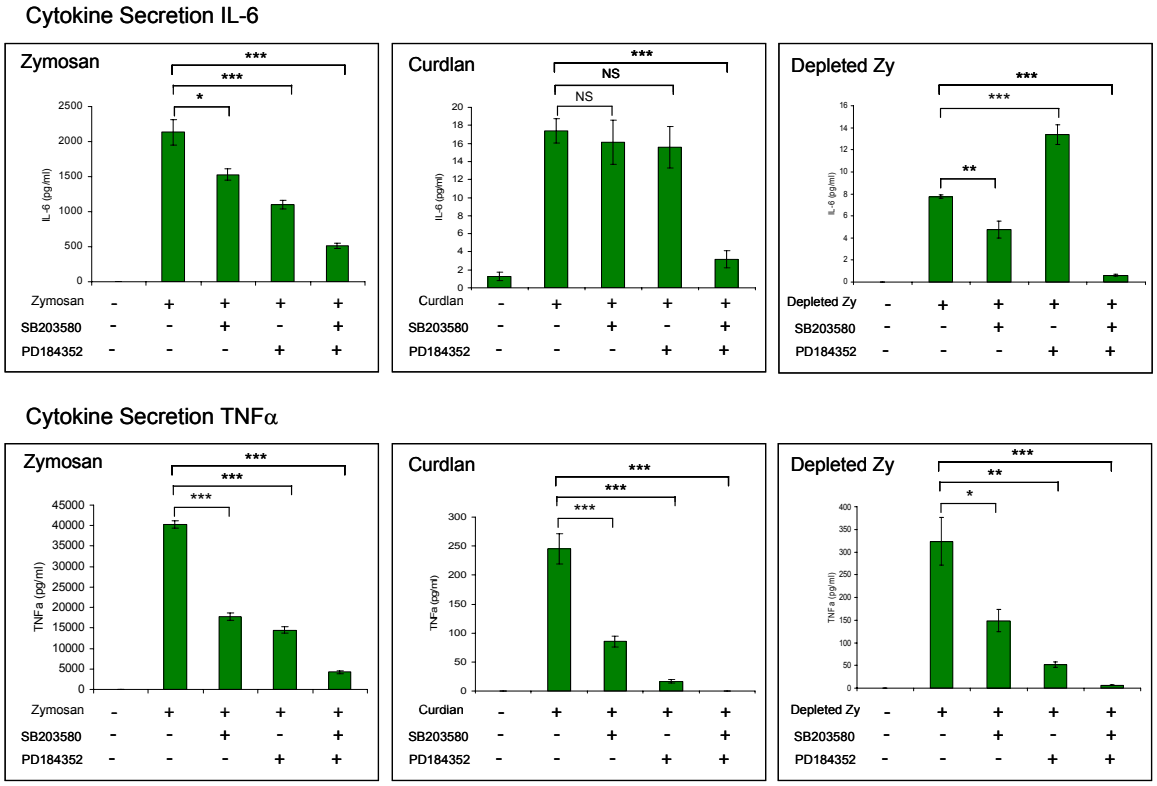
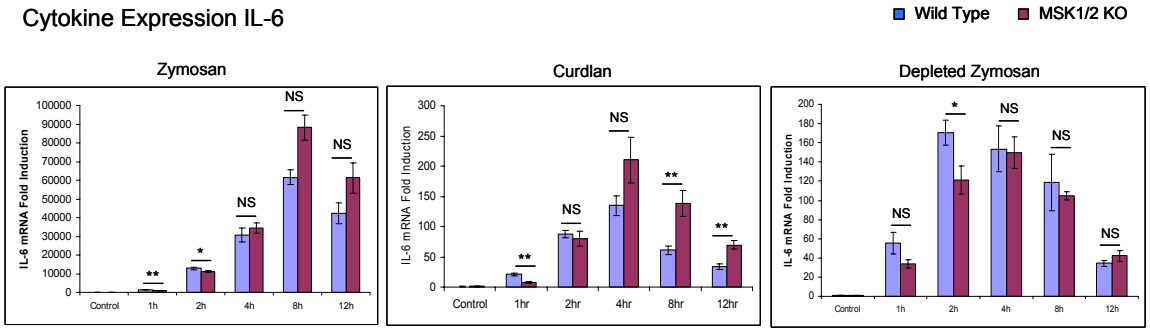


Figure 30 Cytokine Secretion in Response to Dectin-1 Ligands in Wild Type BMDMs +/- MAPK Inhibitors. BMDMs incubated for 1 hour with inhibitors prior to stimulation for 8 hours with either 200µg/ml zymosan, 10µg/ml curdlan or 200µg/ml depleted zymosan. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



To further study the effect of MSK1/2 deficiency on pro-inflammatory cytokine production, I stimulated wild type and MSK1/2 knockout BMDMs with either zymosan, curdlan or depleted zymosan (Figure 31 and Figure 32). Although in the MSK1/2 knockout BMDMs I have shown that less IL-10 is secreted than the wild type in response to Dectin-1 ligands, Figure 31 shows that in the MSK1/2 knockout BMDMs there is a trend towards increased expression of IL-6 and TNFα mRNA in the knockouts, notably at the 4 to 8 hour time points, although this only reached statistical significance in the curdlan stimulated BMDMs. Figure 32 shows that there is no difference between the wild type or MSK1/2 knockout BMDMs in the secreted levels of IL-6 in response to either zymosan, curdlan or depleted zymosan. There is a marginal decrease in the levels of TNFα secreted by the MSK1/2 knockout compared to wild type in response to all 3 stimuli. Overall, this would suggest that IL-10 is much less effective at repressing the effects of Dectin-1 stimulated pro-inflammatory cytokines than it is for TLR induced cytokine production.

Figure 31 Cytokine mRNA Expression in Response to Dectin-1 Ligands in MSK1/2 Wild Type and Knockout BMDMs. BMDMs stimulated for stated times with either 200µg/ml zymosan, 10µg/ml curdlan or 200µg/ml depleted zymosan. a) Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to wild type expression at 0h. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



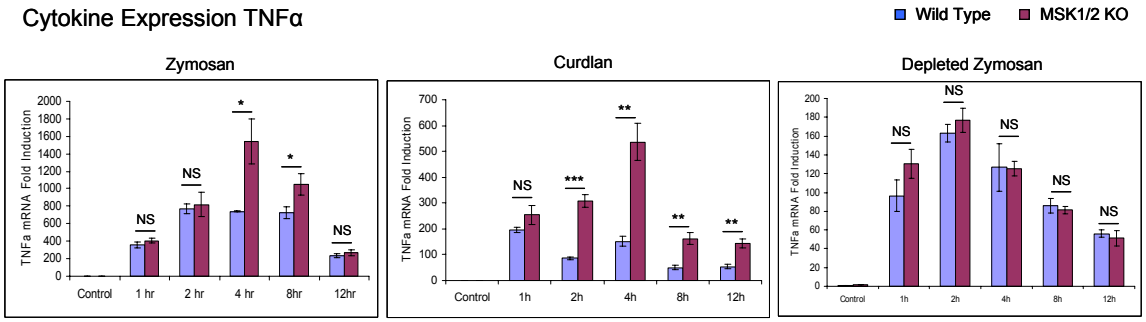
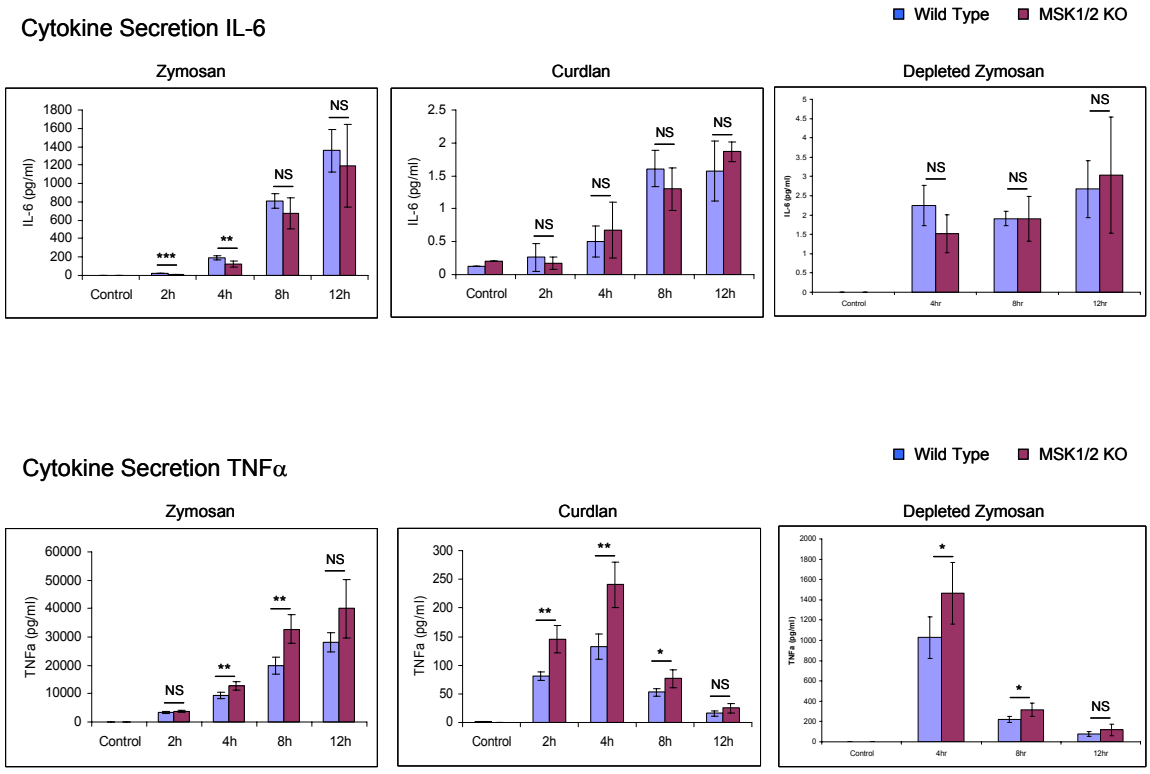


Figure 32 Cytokine Secretion in Response to Dectin-1 Ligands in MSK1/2 Wild Type and Knockout BMDMs. BMDMs stimulated for stated times with either 200µg/ml zymosan, 10µg/ml curdlan or 200µg/ml depleted zymosan. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

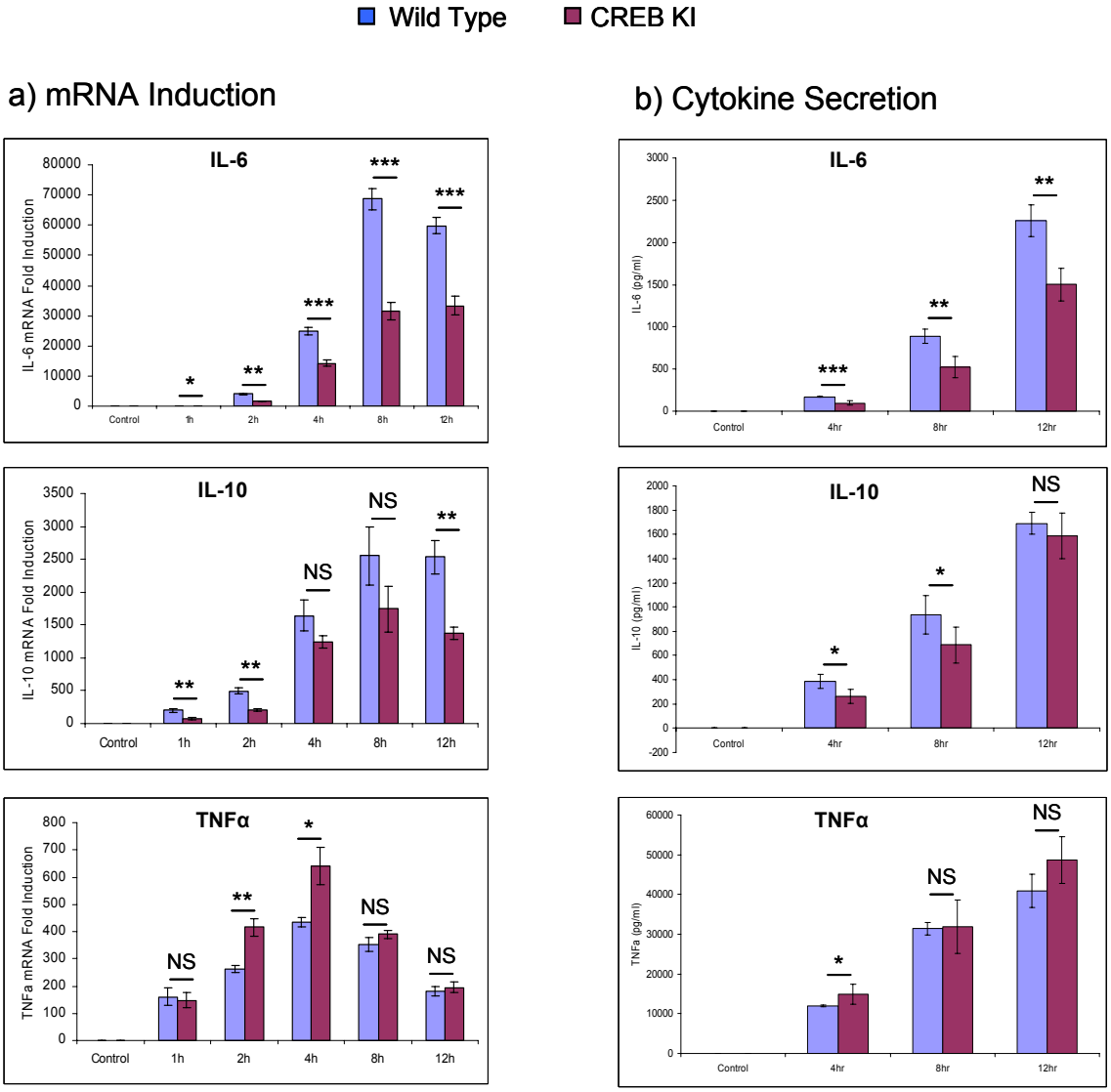


4.5.3 MSK1/2 and CREB

It has been shown that in response to LPS MSK1/2 regulate IL-10 transcription in part through the phosphorylation of CREB. It has been shown that CREB binds to the IL-10 promoter and that CREB phosphorylation at Ser133 is required for maximal IL-10 transcription (Ananieva, Darragh et al. 2008). It is therefore possible that the effects of MSK1/2 knockout we observe are due to the absence of CREB phosphorylation, as figure 21 has shown that the transcription factors CREB and ATF1 are not activated in the absence of MSK1/2.

This was investigated further using BMDMs derived from CREB Serine133 knock-in mice. Total knockout of CREB in mice is embryonically lethal, and as such the best option for our study is the mutation of serine 133, which allows study of MSK1/2 function while the serine 133 independent functions of CREB remain unaffected. Figure 33 shows a zymosan time course in CREB wild type and Serine133 knock-in BMDMs. I show that the CREB knock-in BMDMs do express and secrete marginally less IL-10 and slightly more TNFα than the wild type BMDMs, although the differences between wild type and CREB knock-in BMDMs are not as drastic as those seen between wild type and MSK1/2 knockout BMDMs. Further, these results are not straight forward as the knock-ins unexpectedly express and secrete less IL-6 than the wild types.

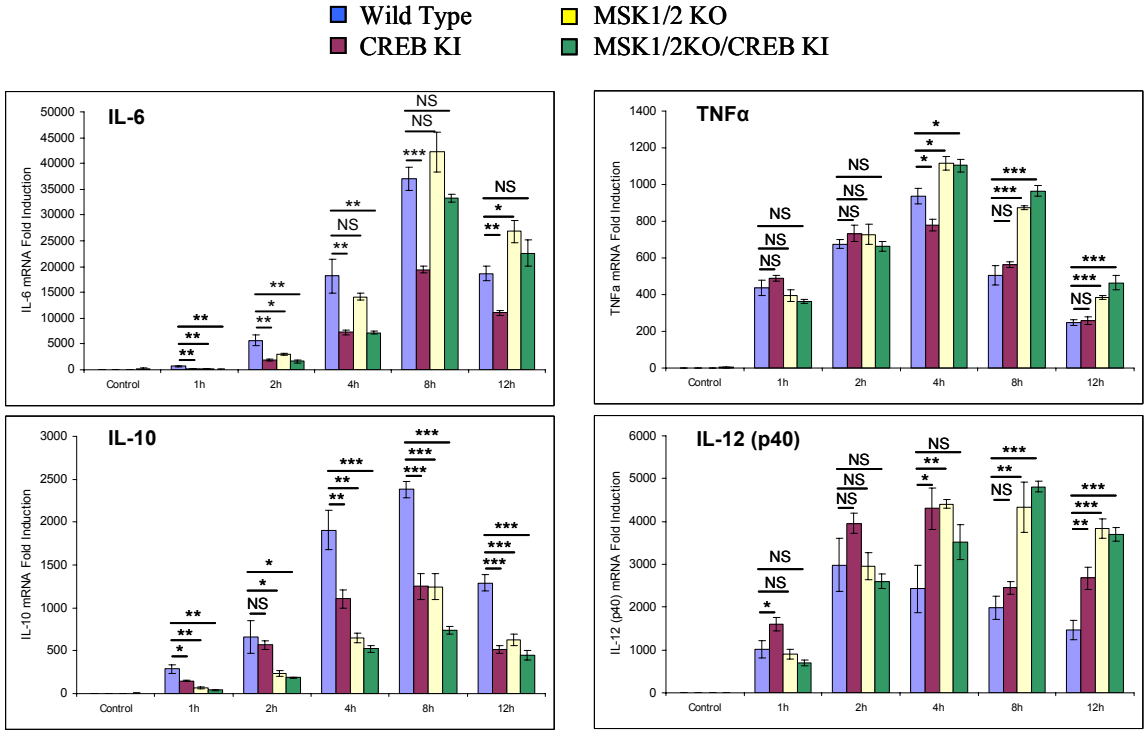
Figure 33 a) Cytokine mRNA Expression and b) Cytokine Secretion in Response to Zymosan in CREB Wild Type and Knock-in BMDMs. BMDMs stimulated for stated times with 200µg/ml zymosan. a) Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to wild type expression at 0h. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



To try and clarify the involvement of CREB in the MSK1/2 knockout cytokine profile we used a combination of wild type, MSK1/2 knock out, CREB knock-in and MSK1/2/CREB triple mice. Figure 34 shows a zymosan time course in all four genotypes, and we see that the mRNA for the pro-inflammatory cytokines TNFα and IL-12 (p40) are, at the later time, points more highly expressed in the MSK1/2 knockout and the MSK1/2 KO/CREB KI triple than in the single CREB knock-in or wild type. This maybe in part explained by the finding that, at all but 1 time point, the MSK1/2 knockout and the triple knockout genotypes express less IL-10 that the CREB knock-in, although the CREB knock-in does still show a significant reduction in expression of IL-10 when compared to the wild type. One possible explanation for these findings is that MSK1/2 is affecting another transcription factor other than CREB, for example ATF1, which is having an additional affect that that of just CREB alone.

Again though, we see that the CREB knock-in expresses significantly less IL-6 than the wild type, and the MSK1/2 knockout and triple knock out genotypes both produce the same or slightly more IL-6 than the wild type. While a cre site has been reported in the IL-6 promoter, it is not clear why the CREB Ser133A knock-in would have a greater effect than the MSK1/2 knockout.

Figure 34 Cytokine mRNA Expression in Response to Zymosan in WT, CREB KI, MSK1/2 KO and MSK1/2 KO/CREB KI BMDMs. BMDMs stimulated for stated times with 200µg/ml zymosan. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to wild type expression at 0h. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



4.5.4 MSK1/2 and IL-10

IL-10 is an important anti-inflammatory cytokine that is known to suppress TLR induced TNFα, IL-6 and IL-12 production in macrophages. As I have shown that MSK1/2 regulates Dectin-1 induced IL-10 production, I looked to see if the effect of MSK1/2 knockout on pro-inflammatory cytokines could be explained by changes in IL-10. To do this, I used BMDMs from IL-10 knockout and IL-10/MSK1/2 triple knockout mice (Figure 35 and 36). These experiments show that in response to LPS the IL-10 knockout BMDMs expressed significantly more IL-6, TNFα, IL-12 (p40) and IL-1β than the wild type BMDMs, whilst the MSK1/2 knockout BMDMs expressed these pro-inflammatory cytokines at a level less than the IL-10 knockout alone, but more than the wild type BMDMs. The IL-10/MSK1/2 triple knockout BMDMs expressed these pro-inflammatory cytokines at a level similar to that of the IL-10 knockout alone. As LPS signals through TLR4, you may expect the TLR2 agonist Pam3CSK4 to show a similar cytokine expression profile. In fact, there appears to be very little difference in the response of IL-6 between genotypes, whilst IL-12 (p40) shows a slight increase in expression and secretion in all three knockout genotypes over the wild type. TNFα appears to be affected more by the MSK1/2 knockout than the IL-10, as the single IL-10 knockout BMDMs show TNFα expression at a level identical to that of the wild type, whilst the MSK1/2 knockout up regulates TNFα expression compared to the wild type. The IL10/MSK1/2 triple knockout shows a trend towards upregulating TNFα expression, but did not reach statistical significance.

The Dectin-1 and TLR2 agonist zymosan shows a similar pattern of cytokine changes as LPS, but whilst the IL-10 knockout has a big effect on LPS induced IL-6 and TNFα secretion, it only has a minor effect when zymosan is used. Zymosan does cause an increase in the expression TNFα, IL-12 (p40) and IL-6 in the IL-10 knockout and the IL-10/MSK1/2 triple knockout when compared to wild type, whilst the level of induction in the MSK1/2 knockout is greater than wild type, but significantly less than that of the IL-10 knockout genotypes. This supports the hypothesis that the increased levels of pro-inflammatory cytokines seen in response to zymosan in the MSK1/2 knockout BMDMs are likely to be caused by the decrease in IL-10. The fact that the MSK1/2 knockout BMDMs have a reduced level of IL-10 expression and secretion, opposed to a complete absence of IL-10, also explains why when I used the IL-10 knockout BMDMs the level of pro-inflammatory cytokines increase further – as these mice do have a complete absence of all IL-10.

Figure 35 Cytokine mRNA Expression in WT, IL-10 KO, MSK1/2 KO and MSK1/2/ IL-10 Triple KO BMDMs. BMDMs stimulated for 8 hours with either 10µg/ml curdlan, 200µg/ml depleted zymosan, 100ng/ml LPS, 1µg/ml Pam3CSK4 or 200µg/ml zymosan. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to wild type expression at 0h. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

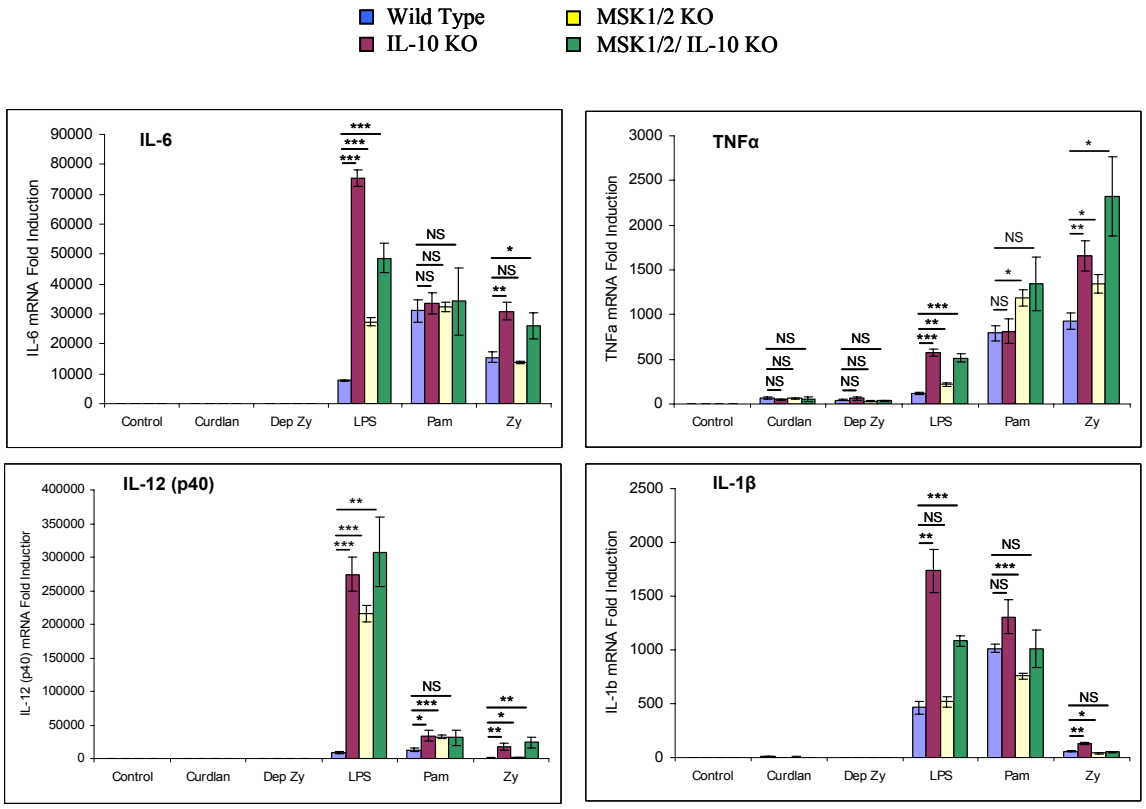
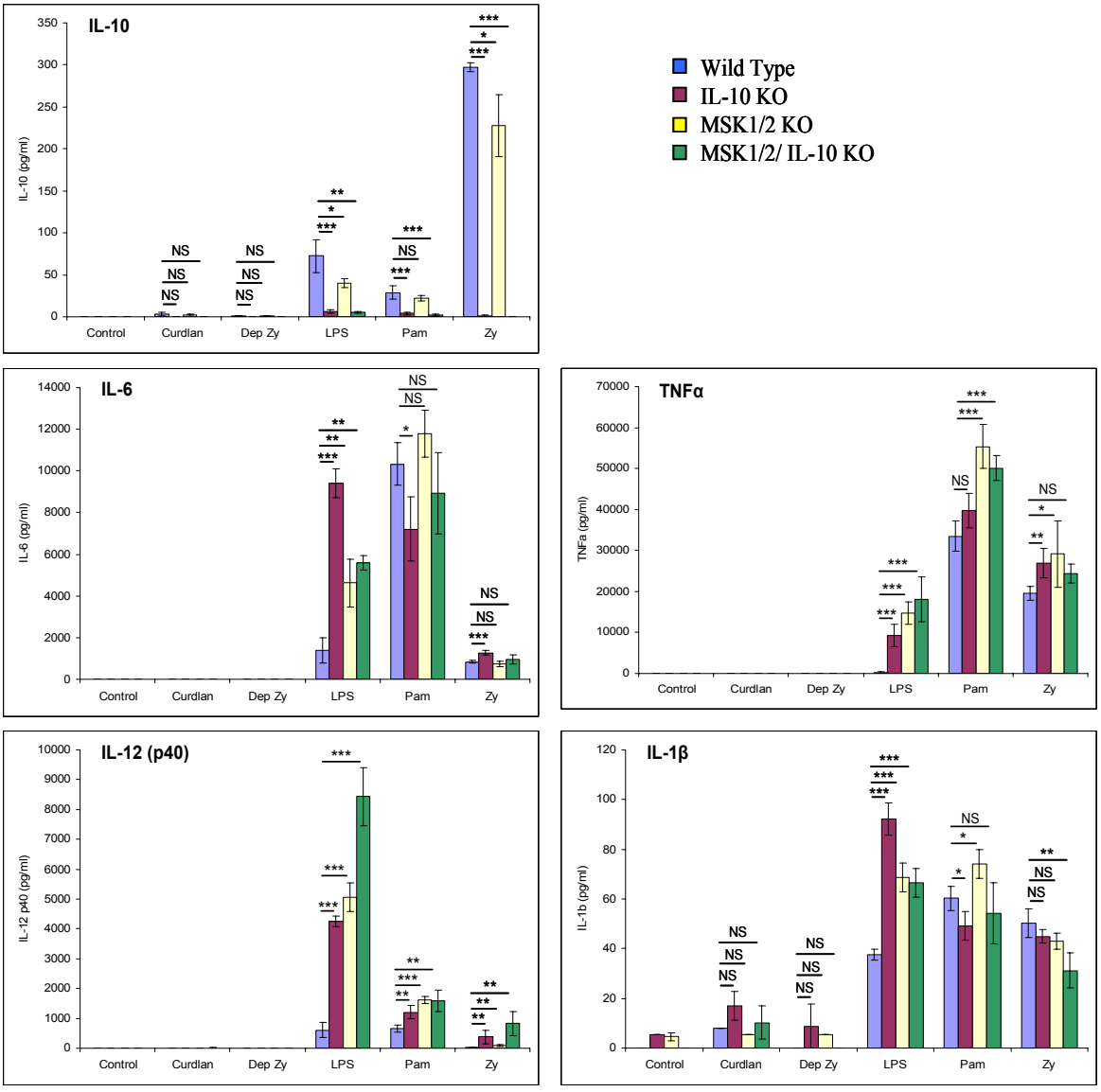


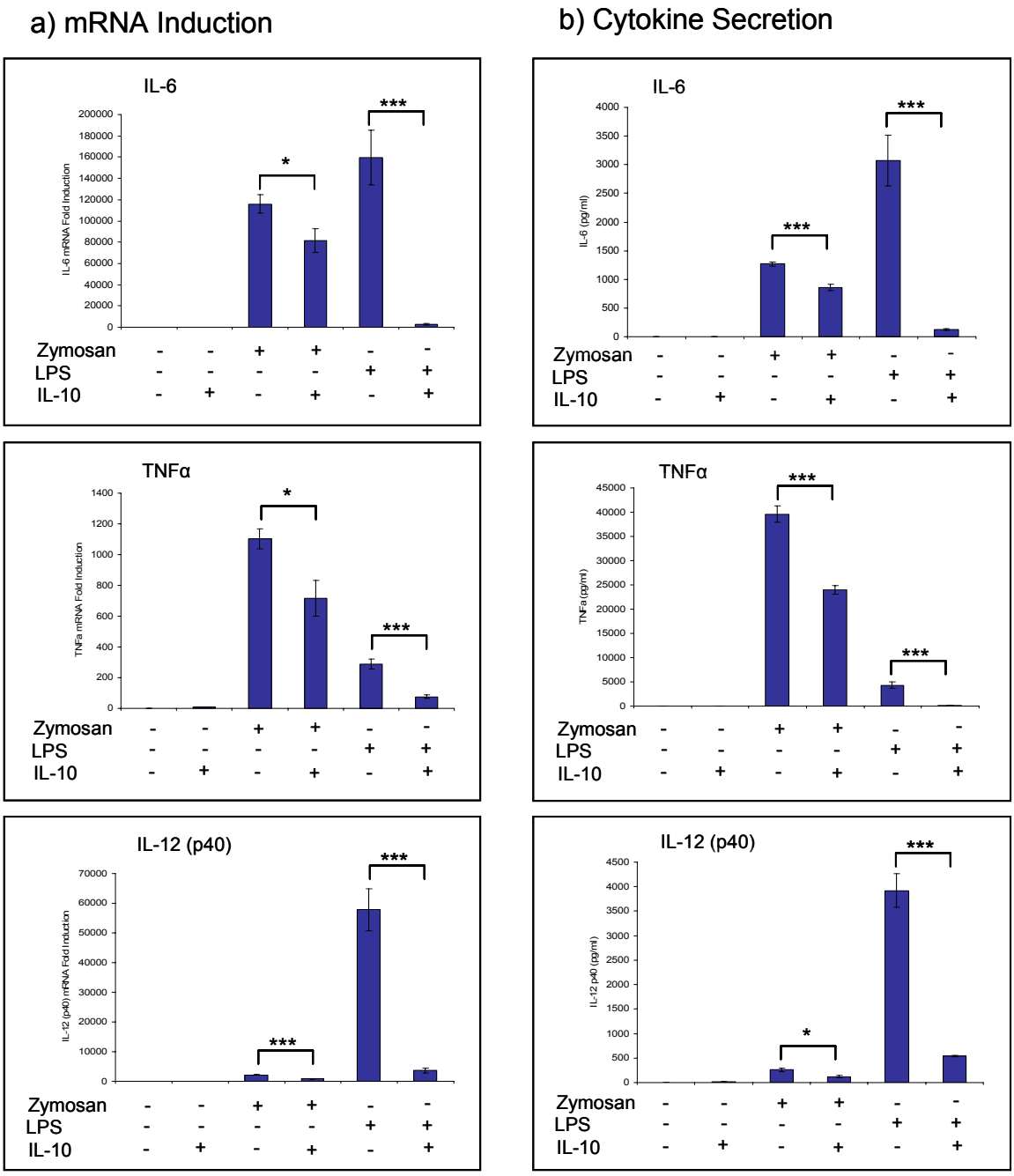
Figure 36 Cytokine Secretion in WT, IL-10 KO, MSK1/2 KO and MSK1/2/ IL-10 Triple KO BMDMs. BMDMs stimulated for 16 hours with either 10µg/ml curdlan, 200µg/ml depleted zymosan, 100ng/ml LPS, 1µg/ml Pam3CSK4 or 200µg/ml zymosan. Error bars show the SD of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



4.6 The Action of IL-10 in Fungal Stimulation of BMDMs

In the MSK1/2 knock out BMDMs, in response to LPS, it has been previously shown that there is a large increase in the pro-inflammatory cytokines TNFα, IL-6 and IL-12 as a direct result of decreased IL-10 expression and secretion (Ananieva, Darragh et al. 2008). As shown in the previous section, MSK1/2 knockout also results in a decrease in IL-10 secretion in response to zymosan. Despite this, MSK1/2 knockout did not result in big increases in zymosan induced TNFα, IL-6 or IL-12 (p40) production. This is in contrast to the LPS results and suggests that IL-10 maybe less able to repress zymosan (or Dectin-1) induced cytokine production relative to LPS/TLR activated BMDMs. In line with this, I showed in figure 36 that while zymosan induces more IL-10 secretion than LPS, the IL-10 knockout had little effect on TNFα or IL-6 production in response to zymosan. To investigate this further I stimulated wild type BMDMs with zymosan or LPS and added exogenous IL-10 (Figure 37). This showed that large amounts of exogenous IL-10 completely suppressed LPS induced TNFα, IL-6 and IL-12 (p40) expression and secretion, however only very modestly suppressed zymosan induced TNFα, IL-6 and IL-12 (p40) expression and secretion.

Figure 37 a) Cytokine mRNA Expression and b) Cytokine Secretion in Response to Zymosan and LPS in +/- IL-10 in Wild Type BMDMs. BMDMs stimulation for 6 hours with 200µg/ml zymosan, 100ng/ml LPS and 100ng/ml exogenous IL-10 as indicated a) Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



4.6.1 IL-10 and STAT Activation

IL-10 exerts its anti-inflammatory effects by inducing the Janus Kinase-STAT3 pathways. Within this signalling pathway, STAT3 plays a crucial role, as without STAT3 IL-10 can not effectively suppress pro-inflammatory cytokine expression (Williams, Bradley et al. 2004). As zymosan induced a high level of IL-10 secretion, but this was unable to repress pro-inflammatory cytokines, I examined the phosphorylation of STAT3 on tyrosine705 as this is the site phosphorylated by JAK1/Tyk2 in response to IL-10.

Figure 38 shows that I achieve much greater STAT3 (tyrosine705) phosphorylation in response to zymosan than to LPS, which is consistent with the higher IL-10 secretion by zymosan (Figure 36). Additionally, I see that STAT1 (tyrosine701) and particularly STAT5 (tyrosine694) are significantly more activated in response to zymosan than LPS. Unfortunately I was unable to assess STAT2 and STAT6, as no adequate antibody was available. As other cytokines, in addition to IL-10, can induce STAT3 phosphorylation, it was important to determine if zymosan induced STAT3 phosphorylation via an IL-10 dependent or independent mechanism.

Figure 39 shows that zymosan stimulation in the IL-10 knockout BMDMs does not cause STAT3 (tyrosine 705) phosphorylation. In line with the reduced IL-10 production in MSK1/2 knockout BMDMs, the MSK1/2 deficient BMDMs showed less zymosan induced STAT3 phosphorylation than was seen in the wild type (Figure 40). Interestingly, STAT1 and STAT5 are still activated in the IL-10 knockouts, though to a lesser extent than the wild types.

To show that the IL-10 knockout BMDMs are still capable of IL-10 induced tyrosine705 STAT3 phosphorylation, I stimulated them with exogenous IL-10. Figure 41 shows that IL-10 knockout BMDMs are able to achieve the same STAT3 (tyrosine705) phosphorylation in response to exogenous IL-10 that wild type BMDMs do.

In summary, zymosan induced IL-10 does not repress TNFα or IL-6 effectively. This is not due to a failure of IL-10 to stimulate BMDMs in the presence of zymosan as STAT3 phosphorylation still occurs.

Figure 38 STAT Activation in Wild Type BMDMs.

BMDMs were stimulated with 200µg/ml zymosan or 100ng/ml LPS as indicated, and incubated for the stated times. Cells were lysed in 1% SDS lysis buffer and levels of the indicated proteins determined by immunoblotting.

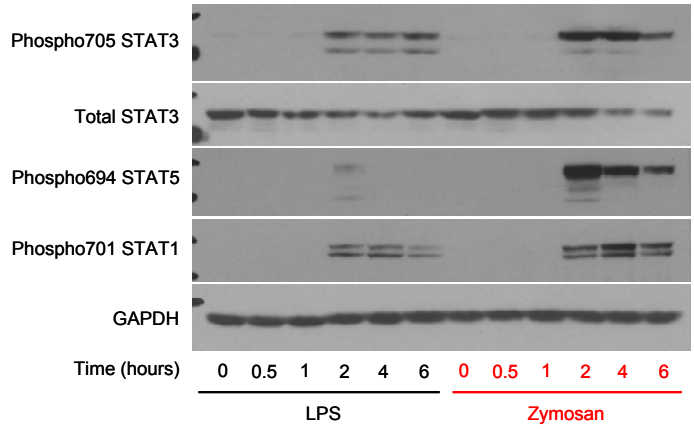


Figure 39 STAT Activation in Wild Type and IL-10 Knockout BMDMs.

BMDMs were stimulated with 200µg/ml zymosan and incubated for the stated times. Cells were lysed in 1% SDS lysis buffer and levels of the indicated proteins determined by immunoblotting.

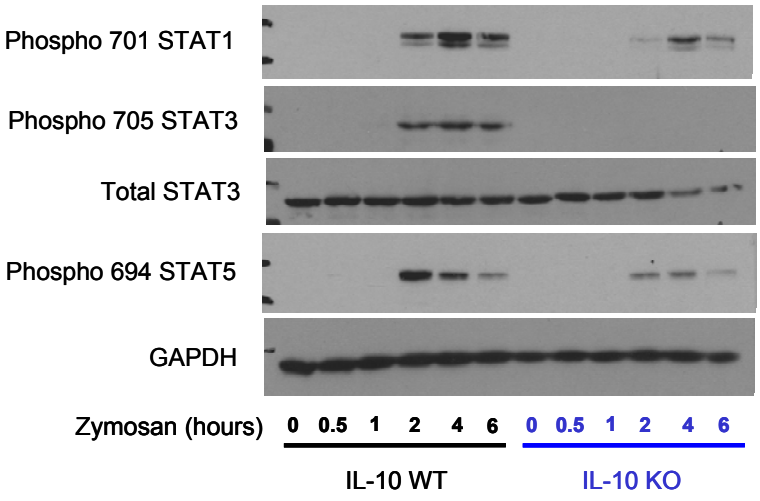


Figure 40 Zymosan Stimulation in Wild Type and MSK1/2 Knockout BMDMs

BMDMs were stimulated with 200µg/ml zymosan for the times stated. The BMDMs were lysed in 1% SDS lysis buffer and the levels of indicated proteins were determined by immunoblotting.

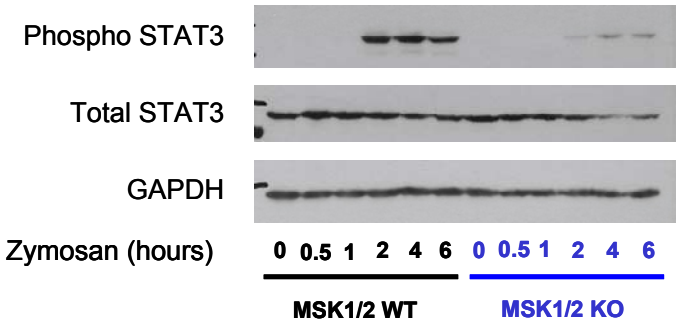
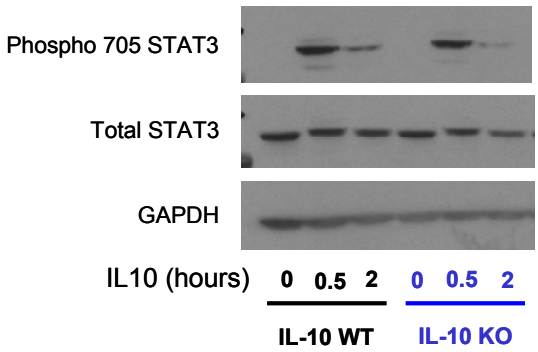


Figure 41 IL-10 Stimulation of Wild Type and IL-10 Knockout BMDMs.

BMDMs were stimulated with 100ng/ml exogenous IL-10 as indicated, and incubated for the stated times. Cells were lysed in 1% SDS lysis buffer and levels of the indicated proteins determined by immunoblotting.



4.6.2 Phagocytosis and IL-10

One obvious difference between stimulations with zymosan and LPS is that zymosan is phagocytosed whilst LPS is not. To examine whether phagocytosis can explain the difference in response to IL-10 produced between zymosan and LPS I used latex beads to be phagocytosed whilst co-stimulating with LPS. Figure 42 shows that the addition of latex beads does not change the level of expression of IL-10 in response to LPS. A combination of latex beads and LPS does reduce the expression of the pro-inflammatory cytokines IL-6, IL-12 p40 and IL-1β relative to LPS alone. LPS in combination with latex beads causes a small increase in expression of TNFα, so it appears phagocytosis contributes to significantly further stimulating TNFα induction, although not to the same level as zymosan stimulation. Phagocytosis alone without PRR stimulation does not appear to induce any cytokine expression.

To investigate whether phagocytosis is the reason why high levels of IL-10 can not repress pro-inflammatory cytokines with zymosan stimulation, we added exogenous IL-10 to the LPS and latex bead co-stimulation. Figure 43 shows that while the addition of beads decreased the ability of LPS to induce IL-6 and IL-12 p40, the addition of exogenous IL-10 was able to repress this further. IL-1β expression however is not decreased with the addition of IL-10, as it also is not with zymosan stimulation. This suggests that it is phagocytosis that prevents the repressive action of IL-10 in IL-1β expression. TNFα expression is only marginally decreased by the addition of IL-10 to the LPS and bead co-stimulated samples, which suggests that phagocytosis maybe in part

responsible for the reduced efficacy of IL-10 in suppressing TNFα in zymosan stimulated BMDMs.

Figure 42 Cytokine mRNA Expression in Response to Zymosan, LPS and Beads in Wild Type BMDMs. BMDMs stimulation for 6 hours with 200µg/ml zymosan, 100ng/ml LPS and 20µl 0.8micron latex beads as indicated. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

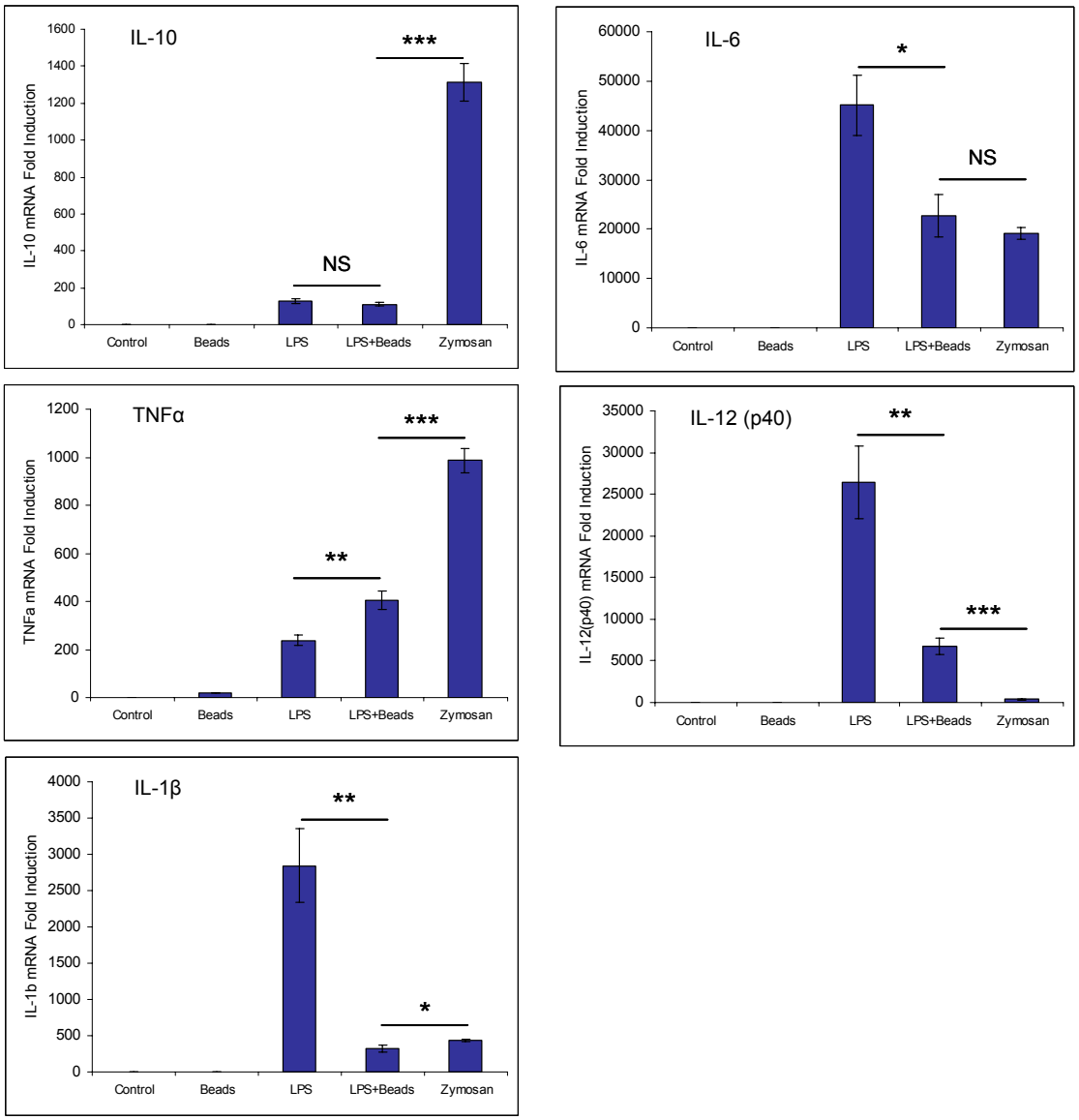
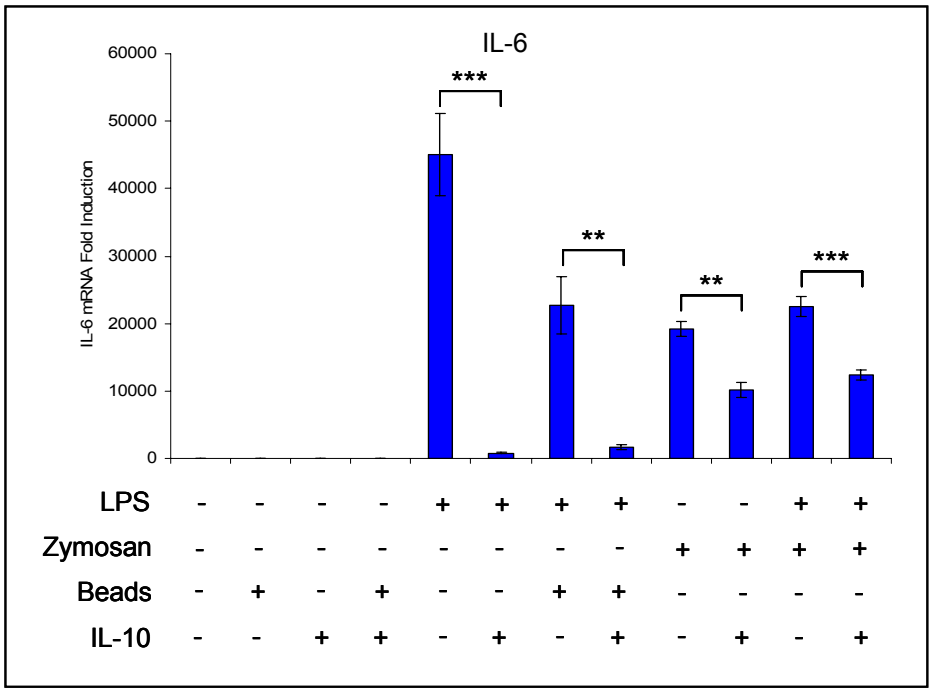
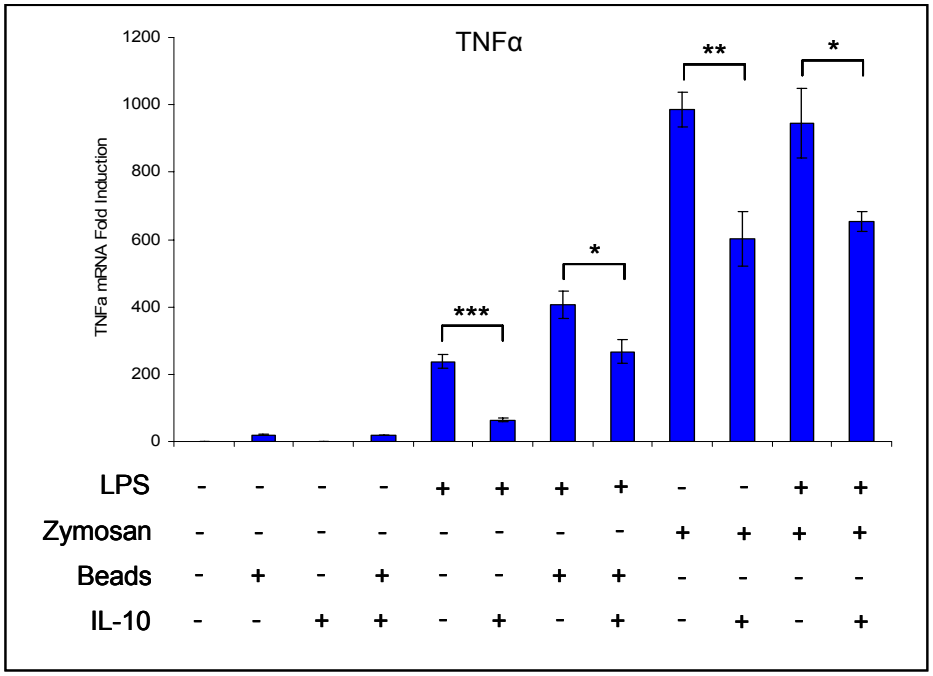
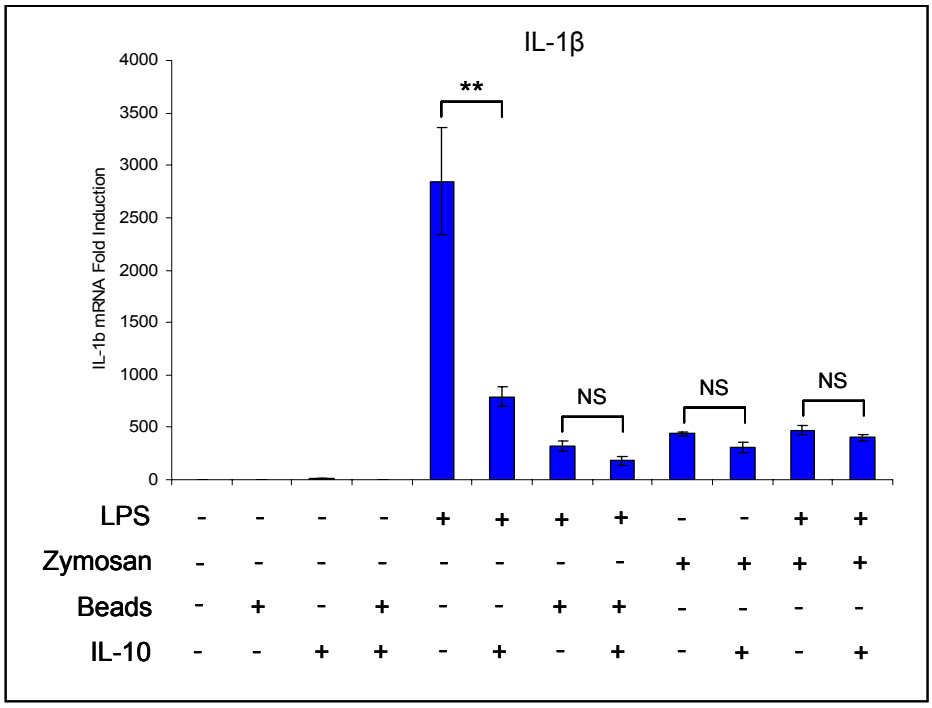


Figure 43 Cytokine mRNA Expression in Response to Zymosan, LPS and Beads +/- IL-10 in Wild Type BMDMs. BMDMs stimulation for 6 hours with 200µg/ml zymosan, 100ng/ml LPS, 20µl 0.8micron latex beads and 100ng/ml IL-10 as indicated. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.






IL-1β
IL-1b mRNA Fold Induction
4000
3500
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1500
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NS
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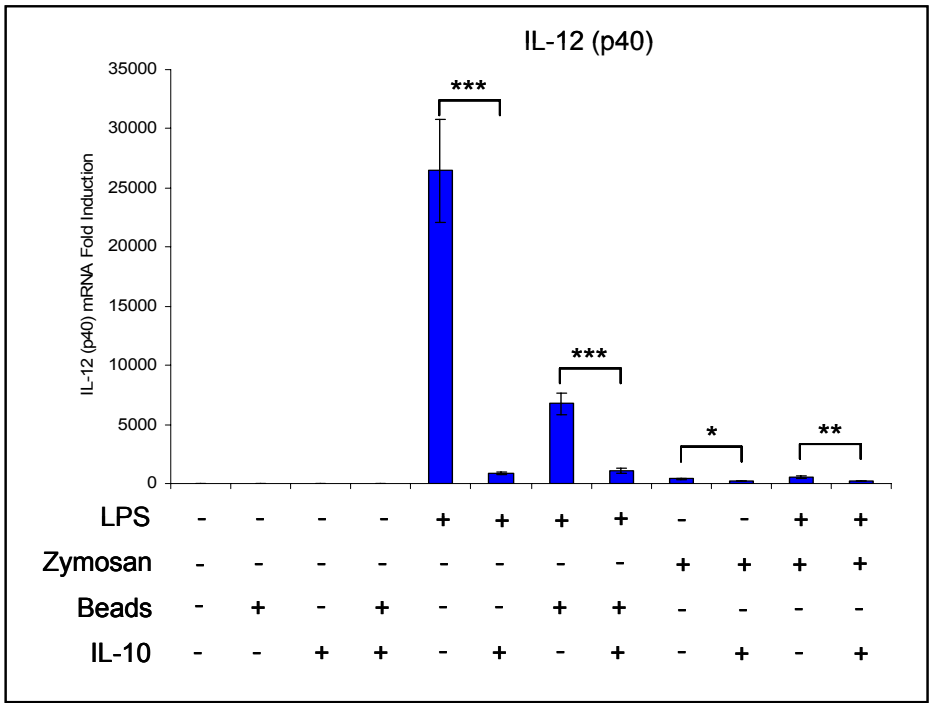
IL-12 (p40)
IL-12 (p40) mRNA Fold Induction
35000
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IL-10 - - + + - + - + - + - +

4.7 Differences between LPS and Zymosan Stimulations

As I have shown, the BMDMs cytokine profile in response to fungal ligands is similar to that of LPS but with several important differences. The first is that, with the exception of TNFα, we see significantly less expression and secretion of the pro-inflammatory cytokines IL-6 and IL-12 (p40); secondly we see much greater induction and secretion of IL-10 in response to fungal ligands, but IL-10 does not seem to repress inflammatory cytokines as it does in LPS stimulated BMDMs. I therefore decided to search for a possible mediator downstream of IL-10 that regulates pro-inflammatory cytokine expression that is different between zymosan and LPS stimulations.

4.7.1 Growth Factor Independent 1

One putative candidate is Growth Factor Independent 1 (Gfi1), a zinc finger protein that functions as a transcriptional repressor. Gfi1 is differentially expressed in haematopoietic stem cells, and plays a large role in B and T cell development (Kazanjian, Gross et al. 2006). Studies in Gfi1 deficient mice show that they are severely neutropenic and accumulate immature monocytes in peripheral blood and bone marrow as they are unable to differentiate into granulocytes, but can differentiate into macrophages (Karsunky, Zeng et al. 2002). Gfi1 deficient macrophages over produce pro-inflammatory cytokines in response to LPS and Gfi1 deficient mice are highly susceptible to LPS induced septic shock (Karsunky, Zeng et al. 2002). It has been shown that macrophages up regulate Gfi1 as a directly as a consequence of TLR4 signalling, and not as a consequence of TLR4 induced inflammatory cytokine production (Moroy, Zeng et al. 2008). The mechanism

for the induction of Gfi1 is unclear; however there is evidence that once the Gfi1 gene is induced and Gfi1 protein is made it forms a complex in the nucleus with the p65 subunit of NF-κB (Sharif-Askari, Vassen et al. 2010). The hypothesis is that this binding prevents p65 binding to NF-κB target gene promoters and as such limits the production of pro-inflammatory cytokines, although this has yet to be proven. It has also been suggested that Gfi1 is able to influence IL-10 signalling by interacting with the STAT3 inhibitor PIAS3 (protein inhibitor of activated STAT). The role of PIAS3 is to bind to activated, phosphorylated STAT3 dimers and block their DNA binding ability (Chung, Liao et al. 1997). It has been shown that Gfi1 is able to interact with PIAS3, resulting in a reduction of the inhibitory effect of PIAS3 on STAT3 activity, and as such Gfi1 could be seen to act as an enhancer of signals transduced by STAT3 (Rodel, Tavassoli et al. 2000).

Gfi1 mRNA was found to be induced by LPS but not zymosan (Figure 44). This upregulation required an increase in transcription, as primary transcript levels were also increased. I did attempt immunoblotting LPS and zymosan treated BMDM samples for Gfi1, but could not find an antibody that worked.

To see if the increase in Gfi1 was due directly to LPS stimulation or indirectly from another protein that was first increased by LPS we performed an LPS time course with and without cycloheximide to block protein synthesis (Figure 45). This suggests that Gfi1 expression is activated by a secondary protein downstream of LPS and not directly by LPS signalling.

As discussed earlier, IL-10 is one protein that is induced by LPS stimulation and can feedback to affect the induction of gene transcription. The level of LPS induced Gfi1 expression was decreased by the addition of exogenous IL-10, shown in Figure 46. This suggests that either IL-10 signalling in some way might cause a decrease in Gfi1 levels either directly, or a consequence of the down regulation of pro-inflammatory cytokines.

Figure 44 Gfi1 mRNA Expression in Response to LPS and Zymosan in Wild Type BMDMs. BMDMs stimulation with 200µg/ml zymosan or 100ng/ml LPS for the stated times. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

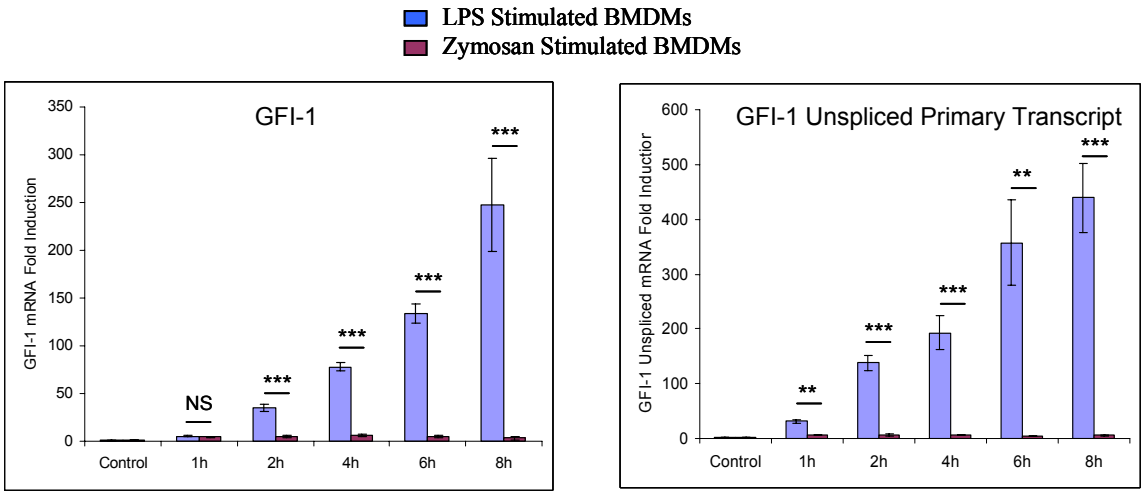


Figure 45 Gfi1 mRNA Expression in Response to LPS +/- Cycloheximide in Wild Type BMDMs. BMDMs were pre-treated for 30 minutes with 10 µg/ml cycloheximide and then stimulated with 100ng/ml LPS as indicated for the stated times. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

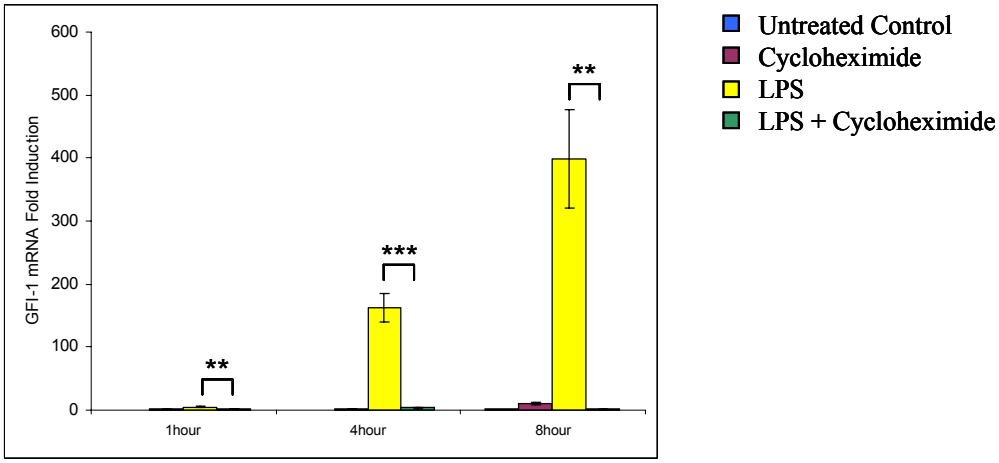
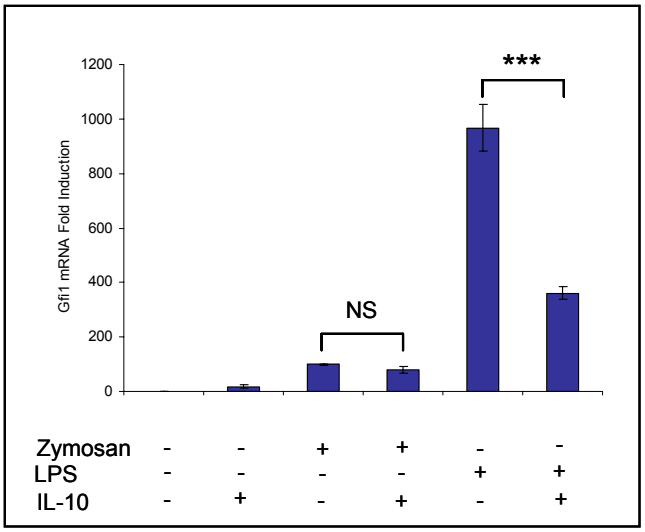


Figure 46 Gfi1 mRNA Expression in Response to LPS and Zymosan +/- IL-10 in Wild Type BMDMs. BMDMs stimulation for 6 hours with 200µg/ml zymosan, 100ng/ml LPS and 100ng/ml exogenous IL-10 as indicated. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



Gfi1 is highly expressed in response to TLR2 and TLR4 stimulation, but is not induced at all in response to zymosan. This is surprising, as we have previously seen zymosan is an incredibly potent activator of TLR2. We must therefore consider what effects on the macrophage that zymosan has in addition to being a TLR2 ligand, such as other PRR involvement (for example Dectin-1 signalling) or the process of phagocytosis that maybe involved in preventing Gfi1 up regulation in response to zymosan stimulation. To examine the hypothesis of phagocytosis preventing the upregulation of Gfi1 in response to zymosan, I stimulated macrophages with LPS and latex beads at the same time to try and mimic the co-activation of TLR and phagocytosis seen with zymosan. Figure 47 shows that the combination of LPS and latex beads did significantly reduce the induction of Gfi1 when compared to LPS alone. This suggests that phagocytosis plays a significant role in the lack of Gfi1 induction in response to zymosan stimulation.

To study Gfi1 further, I planned to knock down the protein using silencing RNA against Gfi1. Unfortunately my initial experiments showed that RAW264.7 cells, unlike BMDMs, do not express Gfi1 in response to LPS (Figure 48). This experiment was not attempted in BMDMs, because at the time we did not have a successful protocol for adequately transfecting primary macrophages.

Figure 47 Gfi1 mRNA Expression in Response to Zymosan, LPS and Beads in Wild Type BMDMs. BMDMs stimulation for 6 hours with 200µg/ml zymosan, 100ng/ml LPS and 20µl 0.8micron latex beads as indicated. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

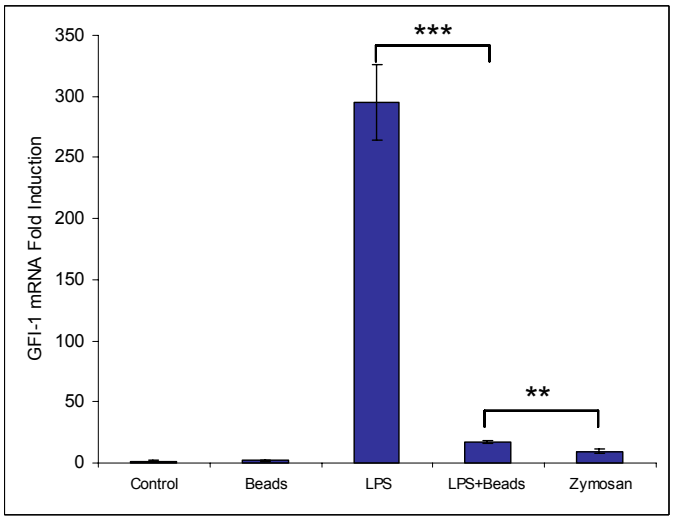
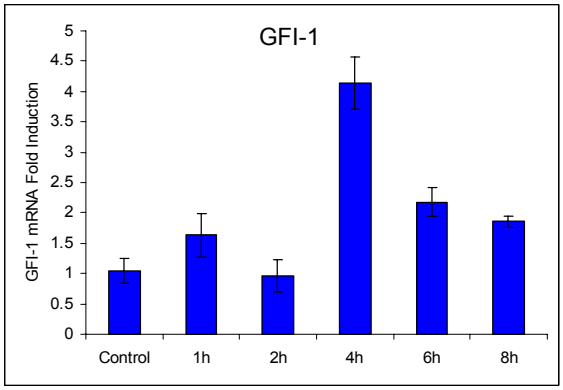
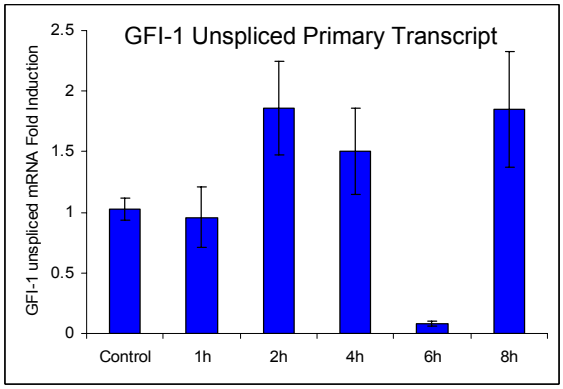


Figure 48 Gfi1 mRNA Expression in Response to LPS in RAW264.7 Macrophage Cell Line. Raw264.7 cells were stimulated with 100ng/ml LPS for the stated times. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction was calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations.





4.7.2 Cyclo-oxgenase 2

Cyclo-oxgenase (COX) is also known as prostaglandin *H*-synthase and is the enzyme responsible for the rate limiting step in pro-inflammatory prostanoid biosynthesis. There are two distinct isoforms of COX, COX1 and COX2. COX1 is present constitutively in many tissues, whilst COX2 is present constitutively in very few tissues but can be induced by a variety of mediators such as cytokines, tumour promoters and bile acids (Seibert, Massferrer et al. 1995). The anti-inflammatory and analgesic effects of traditional nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen and diclofenac, are thought to be primarily due to the inhibition of inducible COX2, whereas the well known gastrointestinal side effects of NSAIDs are thought to be due to inhibition of COX1 (Massferrer, Zweifel et al. 1994). This lead to the development of selective COX2 inhibitors which were hoped to have therapeutic actions similar to traditional NSAIDs but without the GI side effects and are licensed for use in acute pain states and chronic rheumatological conditions (Shi and Klotz 2008).

As COX2 is clearly involved in pro-inflammatory states and its inhibition is an efficacious clinical intervention, I decided to look at the induction of COX2 in response to LPS and zymosan. Figure 49 shows that COX2 is dramatically induced in response to both LPS and zymosan, but LPS induction reaches its peak at about 5 hours and then decreases whilst zymosan induced COX2 expression continues to increase beyond my last time point. This suggests that the initial induction process maybe similar, but there is a continuing stimuli with zymosan. I wondered if it was possible that the continued COX2 expression was in some way connected to the increased levels of IL-10 seen with

zymosan stimulation, as it is reported that IL-10 represses LPS induced COX2 mRNA levels. It is therefore possible that if IL-10 is not working correctly following zymosan stimulation, then it may not repress COX2 mRNA levels. This could be investigated further by examining COX2 mRNA levels following zymosan stimulation in the IL-10 knockout BMDMs.

As COX2 is responsible for prostaglandin production, and as prostaglandin E2 can repress cytokine production by BMDMs, I wondered if high COX2 levels were affecting zymosan induced cytokine production. To examine this further I performed a zymosan time course with and without a COX2 inhibitor (NS398) in wild type BMDMs (Figure 50). This shows that the inhibition of COX2 does not make any significant difference on the induction of IL-10 nor the level of induction of pro-inflammatory cytokines IL-6 or TNFα.

Figure 49 COX2 mRNA Expression in Response to LPS and Zymosan in Wild Type BMDMs. BMDMs were stimulated with 200µg/ml zymosan or 100ng/ml LPS for the stated times. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

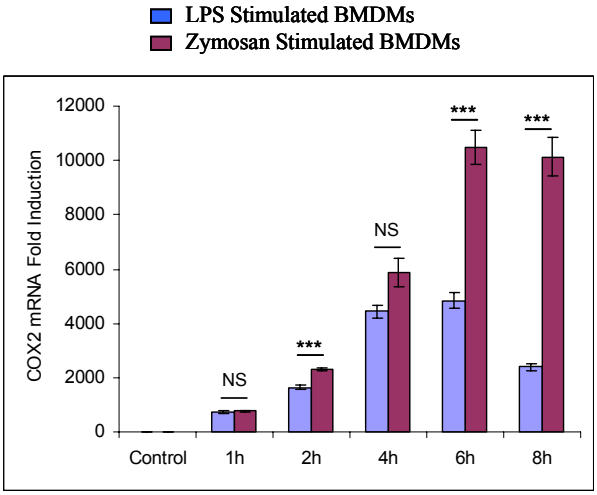
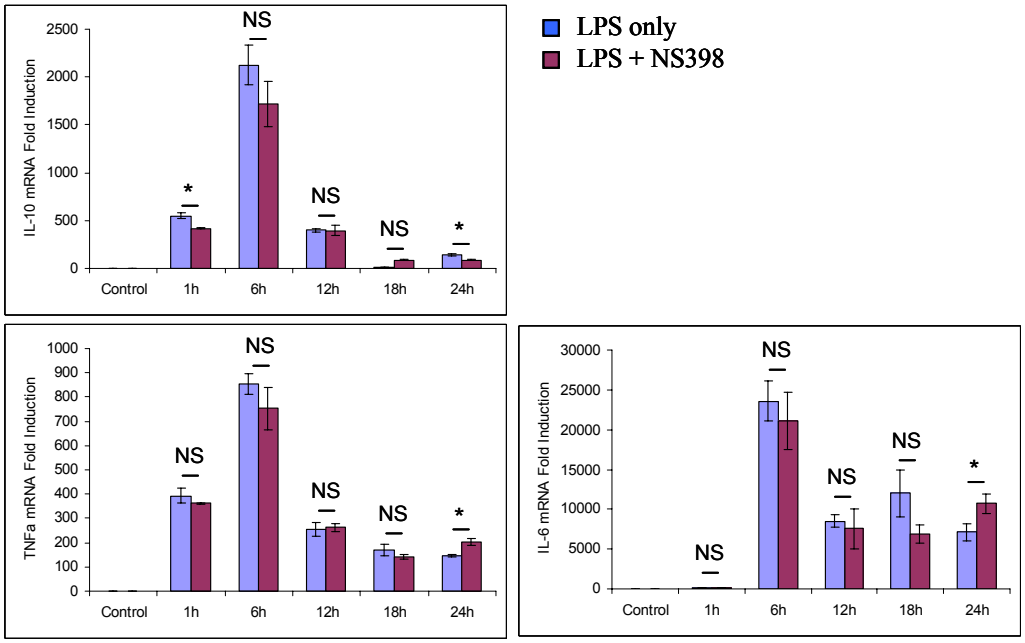


Figure 50 Cytokine mRNA Expression in Response to Zymosan +/- COX2 Inhibitor in WT BMDMs. BMDMs were incubated for 1 hour with 10µM NS398 prior to stimulation with 200µg/ml zymosan for the stated times. a) Quantitative PCR of mRNA, with results normalized to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.



4.8 Fungal Ligands Induce a Regulatory Macrophage Phenotype

Macrophages have to play distinct roles during the inflammation, ranging from a pro-inflammatory function immediately after infection, to an anti-inflammatory function during resolution. In addition, the macrophage response must be tailored to the type of pathogen encountered, and as a result of this macrophages have a very plastic gene expression phenotype that is able to change depending on the exposure to specific stimuli or combinations of stimuli. This allows the macrophage to mount the appropriate response, and has lead to the concept of macrophage polarisation. The most commonly used terms for describing macrophage phenotypes are "Classically Activated Macrophages" (also known as M1) which are activated by TLR stimulation and IFNγ, and "Alternatively Activated Macrophages" (also known as M2) which are predominately activated by IL-4 and IL-13. However, macrophage polarity is now treated more as a spectrum allowing recognition of other subtypes such as regulatory, wound healing and tumour associated macrophages that exhibit distinct but differing characteristics from simply M1 or M2 classic phenotypes (Mosser and Edwards 2008).

It has been well documented that classically activated macrophages, for example macrophages that have been stimulated with LPS, produce high levels of IL-12 and modest levels of IL-10; whilst regulatory macrophages, for example macrophages that have been stimulated with LPS in the presence of immune complexes, produce low levels of IL-12 and high levels of IL-10. The cytokine profile seen in wild type BMDMs following zymosan stimulation shows the typical textbook profile for regulatory

macrophages, so I attempted to investigate whether fungal ligands were able to induce a regulatory macrophage phenotype by examining the presence of regulatory macrophage markers.

To examine my experimental BMDMs I compared the expression of Light and SPHK1, two markers of a regulatory macrophage phenotype (Mosser and Edwards 2008), in response to LPS and zymosan (Figure 51). This demonstrated that BMDMs treated with zymosan express far more Light and SPHK1 mRNA than LPS treated BMDMs. To see if this was due to phagocytosis, I examined the regulatory macrophage marker expression in BMDMs co-stimulated with LPS and latex beads.

Figure 52 shows that phagocytosis does not affect the induction of SPHK1 or LIGHT in response to LPS. We must therefore consider the differences in receptor activation between zymosan and LPS.

To delineate the effects of Dectin-1 and TLR2 activation with zymosan, I examined Light and SPHK1 expression in wild type BMDMs subjected to SYK inhibition, as SYK is required for much of the downstream signalling from Dectin-1. Figure 53 shows that in the presence of SYK inhibitor II or R406 both regulatory macrophage marker expressions are decreased in zymosan stimulated BMDMs. This suggests that the switch to a regulatory macrophage phenotype is Dectin-1 and/or SYK dependent.

Figure 51 Regulatory Macrophage Marker mRNA Expression in Response to LPS and Zymosan in Wild Type BMDMs. BMDMs stimulation with 200µg/ml zymosan or 100ng/ml LPS for the stated times. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

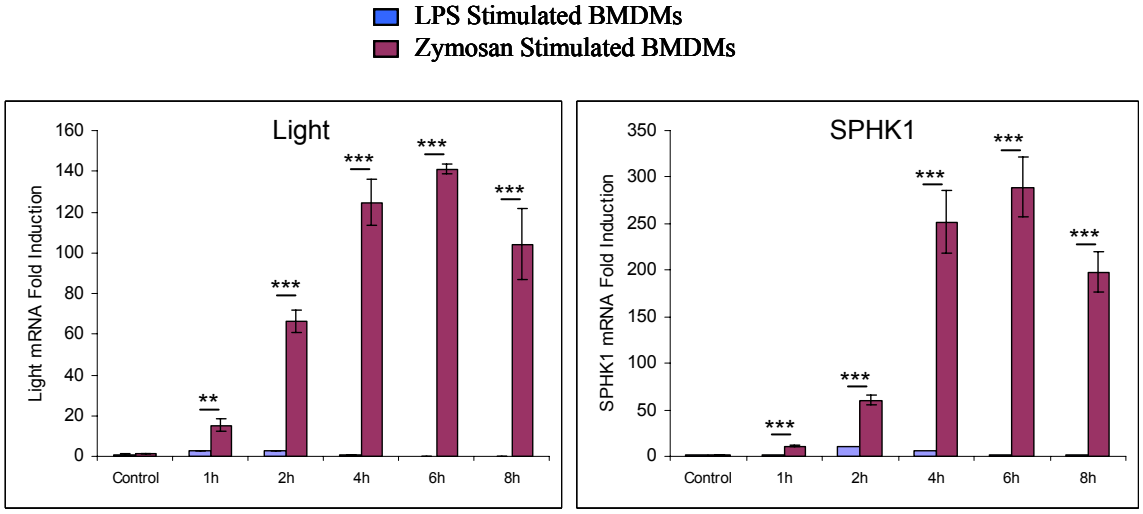


Figure 52 Regulatory Macrophage Markers mRNA Expression in Response to Zymosan, LPS and Beads in Wild Type BMDMs. BMDMs stimulation for 6 hours with 200µg/ml zymosan, 100ng/ml LPS and 20µl 0.8micron latex beads as indicated. Quantitative PCR of mRNA, with results normalised to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * p <0.05; ** p <0.01; *** p <0.001.

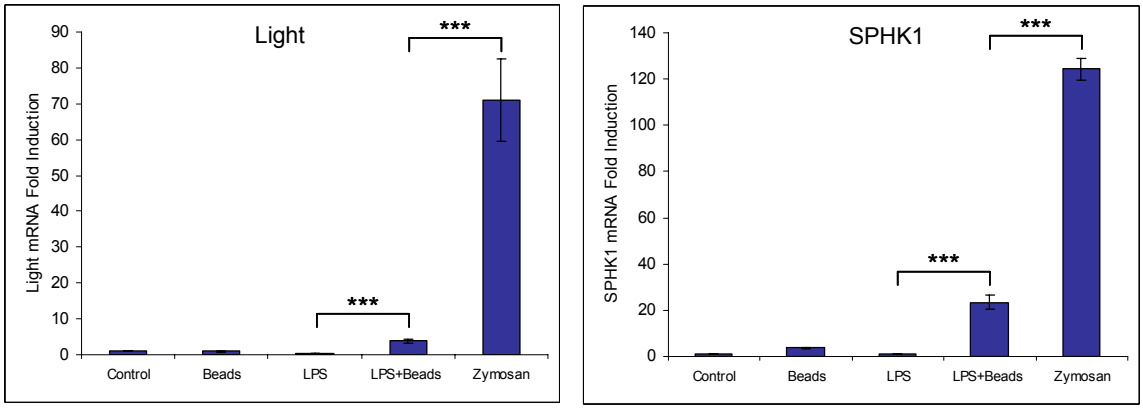
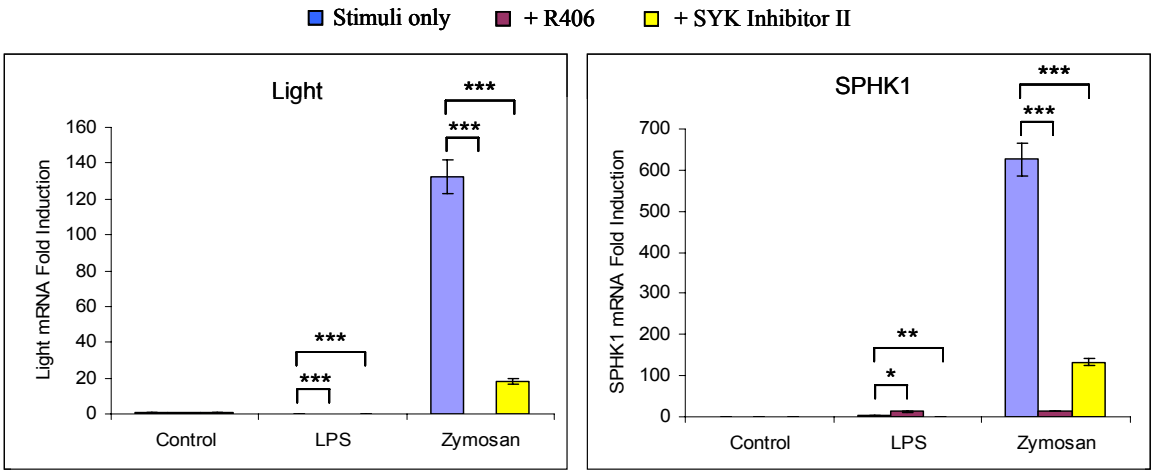


Figure 53 Regulatory Macrophage Markers mRNA Expression in Response to a Variety of Stimuli in Wild Type BMDMs +/- SYK Inhibitors. BMDMs incubated for 1 hour with inhibitors 4μM SYK inhibitor II or 10μM R406 prior to stimulation 8 hours with either 10μg/ml curdlan, 200μg/ml depleted zymosan, 100ng/ml LPS, 1μg/ml Pam3CSK4 or 200μg/ml zymosan. Quantitative PCR of mRNA, with results normalized to expression of 18s RNA and fold induction calculated relative to unstimulated expression. Error bars show the SEM of 4 independent stimulations. A student's t-test (2-sided) was performed on the results; NS is not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



5. Discussion

I have shown that TLR2 and Dectin-1 ligands activate the major MAPK, p38, ERK1/2 and JNK pathways in macrophages. It was observed that the Dectin-1 only stimuli produce an attenuated response in comparison to zymosan. This maybe explained by the synergistic effect described when TLR2 and Dectin-1 signals are combined following zymosan stimulation. Zymosan activation of p38, ERK1/2 and JNK in the Dectin-1 knockout BMDM occurs, but is attenuated in comparison to the wild type. This supports the current theory of receptor synergy for zymosan (Ferwerda, Meyer-Wentrup et al. 2008).

Experiments with the Dectin-1 neutralising antibody and Dectin-1 knockout BMDM are conflicting for curdlan stimulation. While the Dectin-1 neutralising antibody completely prevents depleted zymosan activation of the MAPK pathways, it does not prevent curdlan from activating these pathways. However, in the Dectin-1 knockout BMDM we see no activation of the MAPK pathways following either depleted zymosan or curdlan stimulation. There is currently no clear explanation for this, but one possible suggestion is that the binding of the neutralising antibody to the Dectin-1 receptor may be affected by the stimuli added. For example, it could be possible that the addition of curdlan may disrupt the neutralising antibodies binding, if curdlan has a higher affinity for the Dectin-1 receptor than the neutralising antibody.

To further understand the signalling downstream of Dectin-1 I used SYK inhibitors to attempt to prevent all downstream signalling from Dectin-1. An initial *in vitro* kinase

specificity screen showed that only two of the six tested SYK inhibitors, SYK inhibitor II and R406, actually inhibit SYK significantly and specifically enough to be worth investigating further. This evidence was supported by finding that these were the only two SYK inhibitors that prevented activation of BCR signalling in A20 cells – a process that fundamentally requires SYK. When SYK inhibitors are used in wild type BMDMs we would expect that Dectin-1 only ligands curdlan and depleted zymosan would not be able to activate downstream signalling in the presence of SYK inhibition, but that LPS and to a certain extent zymosan, would be unaffected as these are both strong TLR ligands. In actual fact I show that while SYK inhibitor II is able to prevent signalling in response to Dectin-1 only ligands, R406 causes only a minor decrease in phosphorylation of ERK1/2, p38, p105 and TBK1, and that activation is significantly delayed in the presence of the inhibitor. This maybe due to the stability of R406, although this seems unlikely as R406 works efficaciously *in vivo*, or due to an off target effect on another kinase or phosphatase. AS LPS signals through TLR4, we might not expect to see any major difference in LPS stimulated BMDMs with the use of SYK inhibitors; however it has been reported that SYK is involved in the regulation of LPS induced TLR4 endocytosis (Zanoni, Ostuni et al. 2011). This may explain why both SYK inhibitor II and R406 affect cytokine production following LPS stimulation. However, R406, and not SYK inhibitor II, also caused inhibition of activation of TBK1. Looking at the effect of SYK inhibitor II and R406 on cytokine expression and secretion in wild type BMDMs I show that pro-inflammatory cytokines are marginally reduced in the presence of SYK inhibitor II, but massively inhibited in the presence of R406 in response to TLR2, TLR4 and Dectin-1 stimuli. This does not appear to be IL-10 dependent, as R406 actually

decreases the expression and secretion of IL-10 too. In combination, these findings suggest that possible off target effects of R406 are responsible, as if it was a true consequence of SYK inhibition we would expect to see the same results with SYK inhibitor II, which is arguably a more specific and effective pure SYK inhibitor than R406. Interestingly, R406 is the active metabolite of fostamatinib, a drug currently in phase III clinical trials for the treatment of rheumatoid arthritis (McAdoo and Tam 2011), and it could well be that its efficacy in this setting is due to its effect on another off target kinase, such as TBK1, and not SYK. This could be investigated further by using the TBK1 inhibitor BX795 (Clark, Plater et al. 2009) in combination with the SYK inhibitors to see if there is a synergistic effect, or by using BX795 in the conditional SYK knockout BMDMs.

To investigate signalling further downstream from Dectin-1 I attempted to identify the kinase responsible for ERK1/2 activation. For most stimuli, Raf-1 activates MEK1/2 which activates ERK1/2 (Kolch 2000), however in response to TLR2 signalling the kinase Tpl2 is required to activate MEK1/2, which activates ERK1/2 (Das, Cho et al. 2005). It is therefore possible that Tpl2 maybe involved in ERK1/2 activation in response to Dectin-1 signalling. Using the Tpl2 inhibitor SHN681, I have shown that ERK1/2 phosphorylation is significantly reduced in response to zymosan, and completely blocked in response to depleted zymosan. However, in conflict with these findings are the data from the IkappaB inhibitor BIX02514, which blocks the activation of Tpl2 higher in the signalling pathway. This showed that the Dectin-1 only ligands curdlan and depleted zymosan can still activate ERK1/2 when IkappaB is successfully inhibited with

BIX02514, which suggests that ERK1/2 is activated by a non-Tpl2 dependent mechanism. The IkappaB inhibitor data is supported by my findings that Dectin-1 ligands can still activate ERK1/2 in Abin2 knockout BMDMs. Conclusively, the experiments with the Tpl2 knockout BMDMs show that Dectin-1 signalling results in the same intensity of ERK1/2 activation in wild type BMDMs as it does in the Tpl2 knockout BMDMs, and that the Tpl2 inhibitor SHN681 is still able to inhibit the activation of ERK1/2 in the Tpl2 knockout BMDMs. We suggest therefore that the kinase responsible for ERK1/2 activation downstream of Dectin-1 is not Tpl2, but is an unidentified off target effect of SHN681. It is possible that Raf-1 is the kinase involved in the activation of ERK1/2, however on stimulation of wild type BMDMs with PMA I show that the addition of SHN681 does reduce ERK1/2 phosphorylation, but does not block it completely. This suggests that in cells Raf is inhibited to some degree by SHN681, but not fully, and so another kinase is likely to be involved. To investigate further will be difficult as there are many isoforms of Raf-1, and as it is fundamental in many cellular processes Raf-1 has many self-regulating mechanisms to prevent either its inhibition or stimulation (Hall-Jackson, Goedert et al. 1999).

To examine the signalling pathways even further downstream, we examined the differences between TLR and Dectin-1 signalling in the MSK1/2 knockout model. In the MSK1/2 knockout BMDM we see that in response to zymosan, after 4 hours stimulation, there is an increase in the induction and secretion of pro-inflammatory cytokines IL-12 (p40) and TNFα, with an increase in the expression only of IL-6.This is in keeping with the data published previously on LPS stimulation. Looking at the anti-inflammatory

effectors in response to zymosan we see a significant decrease in the cytokine IL-10, but see no real change in the levels of the MAPK phosphatase DUSP1 in the MSK1/2 knockout (data not shown). Comparing zymosan to LPS (from (Ananieva, Darragh et al. 2008), the levels of DUSP1 induced by LPS or zymosan is of a similar magnitude and the duration of p38 activations are comparable too.

The Dectin-1 only stimulations with curdlan or depleted zymosan in the MSK1/2 knockouts show a very similar picture to zymosan, but with one main difference, in the level of expressed TNFα, which is actually less than the wild type in the MSK1/2 knockouts whilst the level of secreted TNFα is still higher in the knockouts than the wild type.

When we use the inhibitors PD184352 and SB203580 in combination to chemically inhibit MSK1/2, we see a decrease in mRNA expression and cytokine secretion of IL-6, TNFα and IL-10 in the BMDM in response to zymosan, curdlan and depleted zymosan. This enhanced effect of inhibitors over the knockout is not surprising, as ERK1/2 and p38 may regulate cytokine production via MSK1/2 independent mechanisms in addition to the MSK1/2 dependent pathways, or it is highly possible that the inhibitors may also be having off target effects on other kinases involved in cytokine production.

From the fungal stimulated MSK1/2 knockout BMDM Western blots, we see that the transcription factors CREB and ATF1 are not activated in the absence of MSK1/2. It has been shown that in response to LPS CREB phosphorylation at Serine133 and/or ATF1

phosphorylation is/are required for binding to the IL-10 promoter to allow maximal IL-10 transcription (Ananieva, Darragh et al. 2008). I have shown that in response to zymosan the CREB Serine133 knock-in BMDMs express and secrete less IL-10 and more IL-12 (p40) and TNFα than the wild type, but the differences seen are not as drastic as those seen in the MSK1/2 knockout. We were fortunate to be able to use MSK1/2 KO/CREB KI triple BMDMs to examine this further, finding that the action of knocking out MSK1/2 affected more than just CREB activation. Whilst the loss of CREB does account for some of the changes seen with MSK1/2 it is likely that MSK1/2 also activates other transcription factors, the prime candidate being ATF1. To examine this further, we could investigate the role of ATF1 in AFT1 knockout BMDMs, or even see if it is possible to make CREB KI/ATF1 KO mice to examine for other potential transcription factors involved.

Now we must consider how the absence of MSK1/2 may cause these effects on the cytokines produced. It is possible that the increases in IL-12 (p40) and TNFα we observe in the MSK1/2 knockout BMDM is purely down to the decrease in levels of IL-10. This idea is supported by the data from the IL-10 knockout and IL-10/MSK1/2 triple knockout experiments, which also show that in IL-10 deficient BMDMs the production of pro-inflammatory cytokines increases. However, the effect of the MSK1/2 knockout or IL-10 knockout following zymosan stimulation is significantly smaller than the effect following LPS stimulation (Ananieva, Darragh et al. 2008), as it affects fewer pro-inflammatory cytokines and to lesser extent although the levels of IL-10 are decreased to the same level. It is possible therefore that IL-10 does not play such a large role in the suppression

of inflammatory cytokines following zymosan stimulation as it does with LPS. This is illustrated when I saturate wild type BMDMs with exogenous IL-10; I was unable to suppress zymosan induced pro-inflammatory cytokines significantly, whereas with pure TLR agonists, such as LPS or Pam3CSK4, exogenous IL-10 can almost fully suppress pro-inflammatory cytokines. IL-10 exerts its anti-inflammatory effects by inducing the JAK-STAT transcription factor pathways, with STAT3 being fundamentally required for IL-10 to be able to suppress pro-inflammatory cytokine production (Williams, Bradley et al. 2004). In the investigation into why IL-10 does not suppress pro-inflammatory cytokines in response to zymosan, I examined STAT3. Zymosan causes much greater STAT3 phosphorylation than LPS does, which in one way is expected as zymosan induces much more IL-10 than LPS does, but is surprising in another way, as you would imagine that increased STAT3 activation would result in more suppression of pro-inflammatory cytokines. This could be explained if there was a threshold of STAT3 activation that gave maximal effect, and any further activation does not correspond to more suppression, but this does not help explain why LPS suppresses pro-inflammatory cytokines more than zymosan. It is likely is that there are other factors at play. For example, we see that zymosan causes much greater phosphorylation of STAT5 than LPS does, and the possible role of STAT5 in macrophage cytokine production is not clear at present.

One significant difference we see between LPS and zymosan stimulations of macrophages is the level of induced Gfi1. As Gfi1 is a transcriptional repressor that is shown to be important in preventing over production of pro-inflammatory cytokines

(Karsunky, Zeng et al. 2002), it may well play a role in the differences seen between the effects of LPS and zymosan as we see large induction of expression in response to LPS but very little in response to zymosan. PIAS3 is a STAT3 inhibitor, able to bind to activated, phosphorylated STAT3 dimers and blocking their DNA binding ability (Chung, Liao et al. 1997). Gfi1 is reported to interact with PIAS3, reducing the inhibitory effect of PIAS3 on STAT3 activity and enhancing STAT3 signals (Rodel, Tavassoli et al. 2000). In response to zymosan stimulation we see a large amount of STAT3 phosphorylation, but very little Gfi1. It is therefore possible that without Gfi1 to inhibit PIAS3, PIAS3 is binding the activated STAT3 and not allowing it to bind to DNA. This would result in STAT3 being unable to function, and hence no repression of pro-inflammatory cytokine expression would occur. This hypothesis could be tested in future work using Gfi1 knockout BMDMs, as it is a very plausible explanation for the inability of IL-10 to suppress zymosan induced pro-inflammatory cyokines, but leads to the question of why is Gfi1 expressed in response to LPS but not zymosan?

I examined the mechanism of induction of Gfi1 by LPS further, showing that Gfi1 is activated by a secondary protein and not by LPS directly, and that IL-10 does not seem to be involved.

In the quest to find out why IL-10 is ineffective at suppressing pro-inflammatory cytokines in response to zymosan stimulation we must remember that in fungal stimulation the particles undergo phagocytosis by the macrophage. It is entirely possible therefore that while the macrophage is full of pathogen it is not in the cells best interest to

resolve inflammation. To examine this hypothesis further we compared zymosan stimulation to a co-stimulation with latex beads and LPS. We found that phagocytosis and LPS does change the profile of expression of pro-inflammatory cytokines IL-6 and IL-12 p40, causing their levels to be reduced to the level of zymosan expressions, however unlike zymosan stimulation the addition of exogenous IL-10 still did significantly suppress these cytokines. This suggests a mechanism that is not simply the process of phagocytosis and concomitant TLR stimulation, and if I were to investigate further I would use LPS coated latex beads, as I have not accounted for any intra-phagosomal signalling that maybe occurring when zymosan particles used. However, the findings with TNFα are interesting as LPS in combination with latex beads causes an increase in expression of TNFα, so it appears phagocytosis contributes to significantly further stimulating TNFα induction, although not to the same level as zymosan stimulation. In the same fashion, IL-1β is reduced to the level of zymosan induction in the presence of LPS and latex beads, and both TNFα and IL-1β inductions are unresponsive to the addition of exogenous IL-10. It is therefore possible that the process of phagocytosis is involved in IL-1β and TNFα expressions.

In examining the different macrophage phenotypes induced by LPS and fungal signalling, we found that activation of Dectin-1 in a SYK dependent fashion resulted macrophage switching to a regulatory macrophage phenotype. This is very interesting, as regulatory macrophages are thought of as essentially anti-inflammatory, playing a role in resolving inflammation whilst still retaining the capacity for production of pro-inflammatory cytokines and adaptive recruitment if required (Mosser and Edwards 2008). If fungal

pathogens do promote a regulatory macrophage phenotype this may help explain why many people suffer commensal fungal infections that can persist for long periods of time (for example athletes foot) without developing the classical inflammatory signs of infection.

One of the persisting findings during my study is that the Dectin-1 only ligands are less immunogenic than TLR agonists and as such causes a much lower induction of pro-inflammatory cytokines. This may be because *in vivo* the role of initiating a large inflammatory response is due to TLR2, and that Dectin-1 is there not only to enhance this response, but also to provide a level of activation in response to commensal fungal pathogens. Perhaps this is why we see that knocking out Dectin-1 makes little difference to the clinical outcome of mice infected with virulent pathogens such as *Cryptococcus neoformans* and P*neumocystis carinii* (Saijo, Fujikado et al. 2007). In these instances the Dectin-1 knockout mice respond in an identical manner to their wild type counterparts. However, when the Dectin-1 knockout mice are challenged with fungi that are prevalent in the environment, such as A*spergillus* , they are unable to mount a satisfactory immune response (Werner, Metz et al. 2009). This is supported by the known human mutation in Dectin-1, as the patients whom have the mutation are at a much increase susceptibility to development of mucosal and cutaneous candidiasis, but do not succumb to systemic fungal infection (Ferwerda, Ferwerda et al. 2009). In conclusion, it seems that Dectin-1 is essential for initial anti-fungal defences at colonisation sites, but once the fungal pathogen enters the body systemically it is recognised and destroyed, either by innate recognition (especially via TLR2) or by the adaptive immune system.

6. References

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